Social Behaviors of *Pseudomonas putida* IsoF

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Members of the genus *Pseudomonas* are metabolically and physiologically extremely versatile. They inhabit a wide variety of habitats, including diverse terrestrial and aquatic niches. Some strains are of great interest because of their importance to cause plant and human diseases, as well as for their potential in biotechnological applications The diverse life styles of *Pseudomonas* sp. and the complexity of their interactions with multiple hosts have been the subject of numerous studies.

In nature, most of bacteria live in close association with surfaces as complex communities referred to as biofilms. Bacteria growing in biofilms are usually more resistant to hostile conditions than those growing planktonically. Cell densities are very high in biofilm communities, and since the biofilm matrix also acts as a diffusion barrier for signal molecules, biofilms represent an ideal environment for the induction of cell-to-cell communication systems, generally named quorum sensing (QS) systems. The term QS is generally used to describe the phenomenon that bacteria are capable of perceiving and responding to self-generated signal molecules to coordinate their behavior at the group level. Apart from signaling molecules, pseudomonads are known to produce a variety of bioactive compounds, including biosurfactants, which contribute to their niche adaptation, e.g. bacterial competition or associations with certain plant and animal hosts. Bacteria face a constant battle for space and resources. On account of this rivalry they have evolved numerous strategies to deal with competitors. Phenotypic traits such as biofilm formation, cell-to-cell communication, secondary metabolites production, and bacterial competition are commonly associated with the life style of these bacteria and their study may contribute to a better understanding of their success in the environment.

The focus of this thesis was to investigate: i) the link between QS and biofilm formation, ii) biosurfactant production, and iii) bacterial competition, using the plant-promoting bacterium, *Pseudomonas putida* IsoF as a model organism. My work showed that QS signals (*N*-acyl-homoserine lactones, AHLs) are stochastically produced at early stages of *P. putida* biofilms, and act mainly as self-regulatory signals, triggering asocial motility of induced cells out of microcolonies**.** These findings broaden the perspective on QS by showing that AHLs can control the expression of asocial (self-directed) traits, and that heterogeneity in QS can serve as a mechanism to drive phenotypic heterogeneity in self-directed behavior. In addition I

found that two QS-regulated loci are responsible for biosynthesis of the cyclic lipopeptide (CLPs) biosurfactants, putisolvin I and II. One locus encodes the fatty acid CoA ligase PpuA which is required to attach the hexanoic acid chain to the peptide moiety synthesized by the *psoABC* operon. Finally I found that *P. putida* IsoF antagonizes several proteobacterial species by the aid of a type six secretion system (T6SS) and show that this antagonism is particularly effective in mixed-species biofilms.

Zusammenfassung

Mitglieder des Genus *Pseudomonas* sind metabolisch und physiologisch sehr vielseitig. Dadurch können sie eine Vielzahl an unterschiedlichen Habitaten besiedeln, wie etwa verschiedene terrestrische und aquatische Nischen. Einige Stämme sind aufgrund ihres Potenzials zur biotechnologischen Anwendung von großem Interesse, andere wiederum da sie Krankheitserreger von Pflanzen und Menschen sind. Die unterschiedlichen Lebensweisen von *Pseudomonas sp*. sowie die Komplexität ihrer Interaktionen mit verschiedenen Wirtsorganismen wurden schon in einer Vielzahl von Studien untersucht.

In der Umwelt leben die meisten Bakterien in enger Assoziation mit Oberflächen als komplexe Gemeinschaften, so genannten Biofilmen. Bakterien in Biofilmen sind normalerweise resistenter gegenüber feindlichen Lebensbedingungen als planktonisch lebende Bakterien. Da die Zelldichte in Biofilmgemeinschaften enorm hoch ist und gleichzeitig die Biofilmmatrix als Diffusionsbarriere für Signalmoleküle wirkt, stellen Biofilme eine ideale Umgebung für die Induktion von Zell-Zell-Kommunikationssystemen dar, welche als Quorum-sensing (QS) Systeme bezeichnet werden. Die Bezeichnung QS beschreibt das Phänomen, dass Bakterien selbst generierte Signalmoleküle wahrnehmen und darauf reagieren können um ihr Gruppenverhalten zu koordinieren. Es ist bekannt, dass Pseudomonaden neben Signalmolekülen auch eine Vielzahl bioaktiver Verbindungen, wie zum Beispiel Biotenside, produzieren können. Diese tragen zur Nischenadaptation bei, wie etwa beim bakterielle Konkurrenzkampf oder der Assoziation mit bestimmten Pflanzen- und Tierwirten. Bakterien sind einem andauernden Kampf um Raum und Resourcen ausgesetzt weshalb sie unterschiedliche Strategien entwickelt haben um mit ihren Konkurrenten umzugehen. Phänotypische Merkmale wie Biofilmbildung, Zell-Zell-Kommunikation, Produktion von Sekundärmetaboliten und bakterielle Konkurrenz sind gewöhnlich mit der Lebensweise der Bakterien assoziiert. Das Studium dieser Phänotypen kann zum besseren Verständnis des Überlebenserfolgs in der Umwelt beitragen.

Das Hauptaugenmerk dieser Arbeit war die Untersuchung i) der Verbindung zwischen QS und Biofilmbildung, ii) der Biotensidproduktion und iii) bakterieller Konkurrenz anhand des pflanzenwachstumsfördernden Modelorganismus *Pseudomonas putida* IsoF. Meine Arbeit zeigte, dass QS Signale (N-acyl-homoserine lactones, AHLs) stochastisch in den frühen Phasen von *P. putida*

Biofilmen produziert werden und dabei hauptsächlich als selbst regulierende Signale wirken. Diese lösen asoziale Motilität in induzierten Zellen aus, welche daraufhin ihre Mikrokolonien verlassen. Diese Beobachtungen erweitern das Verständnis von QS indem gezeigt wird, dass AHLs die Expression von asozialen (selbst gerichtet) Merkmalen kontrolliert. Des Weiteren wird gezeigt, dass Heterogenität bei QS als Mechanismus zum Antrieb von phänotypischer Heterogenität in selbst gerichtetem Verhalten fungiert. Zusätzlich habe ich herausgefunden, dass zwei QS regulierte Loci für die Biosynthese der zyklischen lipopeptid-Biotenside , Putisolvin I und II verantwortlich sind. Einer der beiden Loci kodiert für die Fettsäure-CoA-Ligase PpuA, welche eine Hexansäurekette an die Peptid-Gruppe von Putisolvin anhängt, welche vom *psoABC* Operon synthetisiert wird. Schlussendlich habe ich noch herausgefunden, dass *P. putida* IsoF mehrere Proteobakterien mittels eines Typ 6 Sekretionssystems (T6SS) bekämpft. Dieser Antagonismus ist in Biofilmen mit gemischten Bakterienspezies besonders effektiv.

1 Introduction

1.1 The genus Pseudomonas

Microbes, particularly bacteria are considered to be the most successful form of life on earth. This feature relies on their ability to adapt and grow in virtually all environments on our planet. Members of the genus *Pseudomonas* are particularly well known for their metabolic and physiologic versatility, which allows them to inhabit a wide variety of habitats (Clarke, 1982), including diverse terrestrial and aquatic niches (Palleroni, 1992). Some strains are also of great interest because of their importance to cause plant and human diseases (Stover et al., 2000) and for their potential in biotechnological applications (Haas and Defago, 2005; Nikel et al., 2014) (Figure 1).

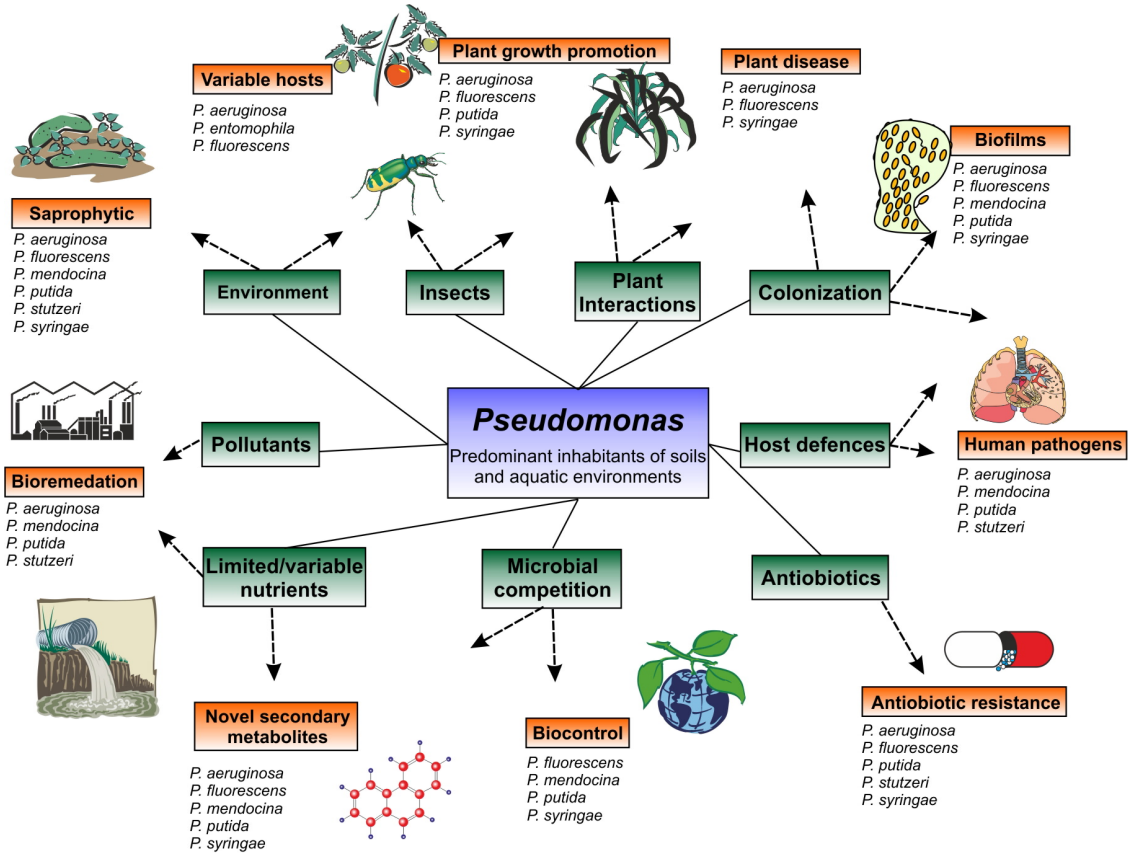

Figure 1. The functional and environmental range of Pseudomonas spp.

The Pseudomonas common ancestor has encountered a wide range of abiotic and biotic environments that has led to the evolution of a multitude of traits and lifestyles with significant overlap among species. Modified from Silby *et al*., 2011.

The taxonomic classification of members of the genus *Pseudomonas* has been a demanding task. Work of Stanier *et al.*, (1966) redefined the genus on the basis of the biochemical capabilities of 267 pseudomonads strains. More recent studies have involved genomic information for a more detailed taxonomic affiliation (Gross and Loper, 2009; Loper et al., 2012; Silby et al., 2011).

1.1.1 General Pseudomonas sp. Characteristics

The diverse life styles of *Pseudomonas* sp. and the complexity of their interactions with multiple hosts have been the subject of numerous studies. Below I present a brief summary of bacterial traits that are commonly associated with pseudomonads, including biofilm formation (and factors involved in this process), cell-to-cell communication, secondary metabolites production, and strategies of bacterial competition in mixed-bacterial populations.

1.1.2 Bacterial Biofilms

In nature, most of bacteria live in close association with surfaces as complex communities referred to as biofilm (Costerton, 2007; Costerton et al., 1995). Biofilms are microbial aggregates growing on solid-liquid or liquid-air interfaces, where the bacterial cells are embedded in a self-generated extracellular polymeric substances (EPS) (Flemming and Wingender, 2010). In addition to abiotic surfaces, biofilms can also be associated with humans (Hall-Stoodley et al., 2004) and plants (Danhorn and Fuqua, 2007). Biofilms have an important ecological role in the production and degradation of organic matter, the degradation of many environmental pollutants and the cycling of nitrogen, sulfur and metals (Davey and O'toole, 2000).

Bacteria growing in biofilms exhibit phenotypic traits that are distinct from those that are expressed during planktonic growth (Hall-Stoodley et al., 2004). Furthermore, the biofilm consortium is intrinsically heterogeneous, partly due to gradients that are form within the matrix (Stewart and Franklin, 2008). Thus, the cells within biofilms are in different physiological states, exhibit diverse phenotypic traits, and express distinct metabolic pathways (Serra et al., 2013; Sternberg et al., 1999; Stewart and Franklin, 2008; Wessel et al., 2014).

Biofilms of human pathogens that grow on tissues or in medical devices can cause persistent infections, that have a very high level of tolerance to antimicrobials (Davies, 2003; Drenkard and Ausubel, 2002; Mah et al., 2003) and are resistant to the host immune system, as the cells are protected against macrophages by the extracellular matrix (Jesaitis et al., 2003). Biofilm cells are also more resistant to disinfectants, metal ions and other toxins (Harrison et al., 2007).

In plant-associated bacteria, the ability to form biofilms is not essential for the interaction (Danhorn and Fuqua, 2007), though it provides advantages to deal with severe environmental conditions face by bacteria in such environments (Monier and Lindow, 2003, 2004). It has been proposed that forming a biofilm is the way to maintain a critical mass of cells, in a specific location for periods sufficient, to initiate beneficial or antagonistic interactions with host (Danhorn and Fuqua, 2007). For instance, the presence of *Pseudomonas syringae* biofilms on leaves microenvironments facilitates the invasion of plant tissue (Melotto et al., 2008)

Biofilms can have a positive influence on its hosts. For example, the enteric microbiota forms a biofilm adjacent to the intestinal mucosa. It is estimated that the human gut harbors 400–1,000 different bacterial species (Mazmanian et al., 2008). Approximately 10^{11} bacterial cells can be found per gram of colon contents (Guarner and Malagelada, 2003). The bacteria that are located in the gut biofilm seem to be less affected by environmental alterations, such as changes in intestinal transit rate and luminal contents, than the planktonic lumenal bacteria (Macfarlane and Dillon, 2007). Plant-beneficial biofilms that colonize different plant tissues promote plant growth by providing nutrients, fixing nitrogen, protecting plants against pathogens, and degrading various pollutants (Danhorn and Fuqua, 2007; Segura and Ramos, 2013). Furthermore, biofilms are especially valuable for the treatment of recalcitrant substances because of their high microbial biomass and their ability to immobilize such compounds (Singh et al., 2006)

The molecular mechanisms involved in the regulation of biofilm formation can vary greatly among species and even between strains of the same species. However, there are some common features that can be distinguished: i) the presence of an extracellular matrix that holds the cells together, which is composed of exopolysaccharides, proteins and nucleic acids; ii) the response to extracellular or self-generated signals; iii) the biofilm provides the bacteria with a physical barrier against an array of environmental stresses including antibiotics and predators.

1.1.2.1 Biofilm formation

Biofilm formation is a complex phenomenon, which involves the integration of environmental and cellular signals into an intricate regulatory network, leading to the adaptation of bacteria to multicellular life on a surface (Hall-Stoodley et al., 2004). The sequence of events involved in the transition from planktonic cells to sessile biofilm cells has been intensively investigated (Sauer et al., 2002; Stoodley et al., 2002). Biofilm formation is considered to be a developmental process in which bacterial cells switch their lifestyle from a nomadic planktonic state to a sessile state. Subsequent proliferation of the cells on the substratum leads to the formation of structured biofilm communities.

Microscopic analysis, mostly of Gram-negative proteobacteria, has led to a general model of biofilm formation involving discrete changes in the spatiotemporal organization of individual cells, from their free-swimming existence to being a member of a surface-associated microbial community (Monds and O'Toole, 2009; O'Toole et al., 2000) (Figure 2).

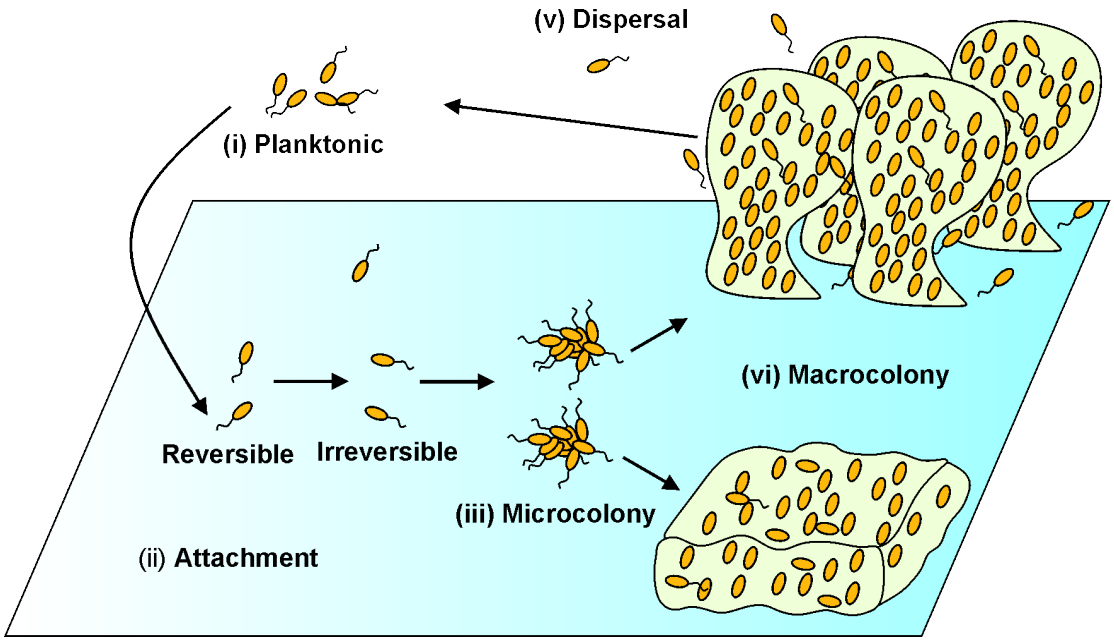

Figure 2. Stages during bacterial biofilm development:

(i) planktonic, (ii) attachment, (iii) microcolony formation, (iv) macrocolony and (v) dispersal. The attachment phase has often been further divided into 'reversible' and 'irreversible' stages, initial attachment to the surface is often weak, with active mechanisms required for cells to develop a more stable association with the surface. Subsequently, discrete cell clusters (microcolonies) are formed either by clonal growth of attached cells or by active translocation of cells across the surface. Microcolonies grow in size to form macrocolonies in a so-called maturation process. Macrocolonies can have different tree-dimensional organizations, e.g. mushroom-like towers separated by fluid-filled voids or flat structures. Cells are held within the macrocolony by a complex matrix, which is in part based on exopolysaccharide. Finally, macrocolonies can disperse, releasing cells from the biofilm. The return of cells to the planktonic phase completes the idealistic picture of the developmental cycle. Together, these five visually demarcated transitions constitute the checkpoints of the biofilm developmental cycle. Modified from Monds and O'Toole, 2009.

The transition from the bacterial planktonic single-cell state to the biofilm state is dependent on self-produced extracellular matrix (Berk et al., 2012; Ma et al., 2009; Newell et al., 2011a). The matrix consists in a conglomeration of different types of biopolymers, (i.e. exopolysaccharides, proteins and nucleic acids) known as extracellular polymeric substances (EPS). EPS forms the scaffold of the three-dimensional architecture of the biofilm and is responsible for adhesion to surfaces and for cohesion of the biofilm (Flemming and Wingender, 2010; Petrova and Sauer, 2012).

1.1.2.2 Bacterial motility and biofilm formation

Bacterial motility enable bacteria to respond to changing environments. Depending on environmental cues, bacteria can remain within the biofilm matrix or leave the biofilm in search for better growth conditions. Prokaryotic cells can move through liquids by swimming (Jarrell and McBride, 2008) or over moist surfaces by swarming, gliding, twitching, sliding, or darting (Harshey, 2003; Henrichsen, 1972).

The involvement of swimming and swarming motility on biofilm formation has been the focus of intensive research in this field. The initiation of biofilm formation through reversible attachment often requires flagella, and motility on a surface can be crucial for biofilm architecture (Eberl et al., 1999; Gibiansky et al., 2010; Shrout et

al., 2006). On the other hand, motility is also involved in the release of bacteria (dispersal) from mature biofilms (Verstraeten et al., 2008) Using biofilms growing in a flow chamber (Christensen et al., 1999; Pamp et al., 2009), it has been observed that after attachment to the surface, *Pseudomonas aeruginosa* cells differentiate into a non-motile and a motile subpopulation. The motile subpopulation translocates on the surfaces of the substratum via switching motility, which depends on both type IV pili and flagella (Klausen et al., 2003, 2003; Gibiansky et al., 2010). The non-motile cells form the stalks and the motile cells the caps of the mushroom-like biofilm structures (Klausen et al., 2003; Shrout et al., 2006).

1.1.3 The role of c-di-GMP in biofilm formation

The secondary messenger bis-(3–5)-cyclic dimeric guanosine monophosphate (c-di-GMP) has been shown an important regulator of the bacterial biofilm lifecycle in several bacteria (Hengge, 2009). In most of cases, high internal levels of c-di-GMP induce biofilm formation as consequence of the production of adhesins and EPS, whereas low c-di-GMP levels down-regulate the production of adhesins and extracellular matrix components and lead to biofilm dispersal and thus are characteristic for planktonic cells (Boyd and O'Toole, 2012). The synthesis and degradation of c-di-GMP in bacteria occur through the opposing activities of diguanylate cyclases (DGCs) with a GGDEF domain and phosphodiesterases (PDEs) with EAL or HD-GYP domains, which are typically modular in nature and contain sensory domains that sense and respond to environmental cues (Romling et al., 2013)

1.2 Quorum sensing

The term quorum sensing (QS) is used to describe the phenomenon that bacteria are capable of perceiving and responding to self-generated signal molecules to coordinate their behavior at the group level (Fuqua et al., 1994). The general consensus is that bacteria trigger the QS response only when their cell density has reached a certain threshold (the “quorum”), upon which the expression of target genes is either activated or repressed. Among the various QS signal molecules identified to date, *N*-acyl-homoserine lactones (AHL) have been investigated to the greatest extent (Whitehead et al., 2001; Williams, 2007), and have been shown to control the expression of a large variety of traits, including bioluminescence,

virulence, symbiosis, different forms of motility, biofilm formation, production of antibiotics and toxins, and conjugation (Bassler and Losick, 2006; Williams et al., 2007).

The first described QS system was described in the marine bacterium *Vibrio fischeri*, and it is considered the paradigm for quorum sensing in most gram-negative bacteria (Waters and Bassler, 2005). Two proteins, LuxI and LuxR, control expression of the luciferase operon (*luxICDABE*) required for light production. LuxI is the AHL synthase, which directs the synthesis of N-3-oxohexanoyl-L-homoserine lactone (3-oxo-C6-HSL). LuxR is the cytoplasmic AHL receptor, which binds the AHL signal molecule and then activates or repesses expression of target genes. As AHLs are produced, they freely diffuse out of the cell and increase in the culture medium with increasing cell density. When the AHL concentration reaches a critical threshold, it binds to LuxR and this complex then in turn activates transcription of the operon encoding the luciferase genes (Figure 3). Cell densities are obviously very high in biofilm communities. Moreover, the biofilm matrix may also act as a diffusion barrier for signal molecules, Hence, cell-to-cell communication systems appear toe be very important regulatory mechanisms for the control of gene expression in biofilms (Parsek and Greenberg, 2005).

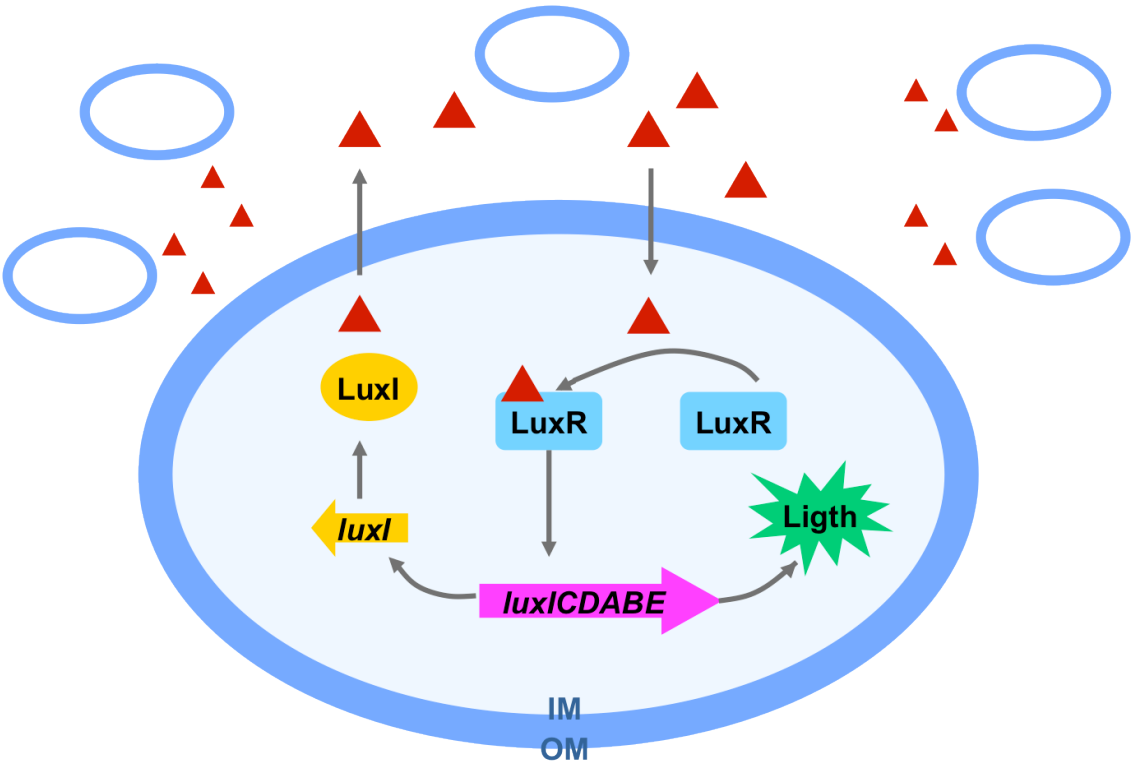

Figure 3. Quorum sensing in *Vibrio fischeri*.

Red triangles indicate the AHL signal molecule that is produced by LuxI. As cell density increases (denoted by light blue ovals) the signal molecule accumulates until a certain threshold is reached, upon which the entire population switches to the "quorum-sensing mode" and produce light. Modified from Waters and Bassler, 2005.

1.3 Production of secondary metabolites

Pseudomonas species produce a variety of bioactive compounds, including siderophores, hydrogen cyanide, 2,4-DAPG, pyrrolnitrin, pyoluteorin, phenazines, 2,5-dialkylresorcinol, quinolones, gluconic acid, rhamnolipids, and various LP antibiotics. Many of these compounds have been demonstrated to be important for the biological control of plant pathogens (Raaijmakers and Mazzola, 2012). Secondary metabolites are often only produced by specific lineages, thereby contributing to their niche adaptation, e.g. associations with certain plant or animal hosts (Gross and Loper, 2009). Consequently, the genetic loci responsible for the biosynthesis of these metabolites are usually in non-conserved genome regions. In fact, *Pseudomonas* sp. biosynthetic gene clusters are considered to be among the most diverse and rapidly evolving genetic elements (Fischbach et al., 2008). The diversity of the secondary metabolites produced by *Pseudomonas* sp. is thought to be based on gene acquisition via horizontal gene transfer (HGT) followed by events of mutation, recombination, and duplication or excision (Jenke-Kodama et al., 2006; Juhas et al., 2009).

1.4 Competition in multispecies environments

Biofilms represent a structured environment which promotes the evolution of specific interactions among the community members (Hansen et al., 2007b). Interactions within these communities can be cooperative (Christensen et al., 2002; Elias and Banin, 2012; Hansen et al., 2007a; Hansen et al., 2007b; Ren et al., 2015) and/or competitive (Foster and Bell, 2012; Oliveira et al., 2014; Rendueles and Ghigo, 2012)

Additionally, natural biofilms normally consist of bacteria belonging to different species (Bolhuis et al., 2014). They have established strategies to either take

advantage of beneficial interactions (Elias and Banin, 2012) or preventing detrimental associations (Rendueles and Ghigo, 2012). Moreover, bacteria face a constant battle for space and resources. On account of this rivalry they have evolved numerous strategies to deal with competitors (Hibbing et al., 2010). Biofilms provide an environment with multiples ecological niches, in this scenario; competition leads the selection for variants, which better colonize these specific niches (Boles et al., 2004; Kassen et al., 2004).

Bacteria, have developed contact-dependent inhibition systems, which depend on effector proteins that affect growth of the targeted cell (Hayes et al., 2010). To date, bacterial secretion machines have been classified into seven main groups referred to as type I through VII. Four of those systems, types III, IV, V, and VI, have been shown to recognize target cells upon cell contact and to initiate a series of events that enhance their survival and growth at the expense of the target cell. These systems are especially attractive for their biological roles in competition within bacterial biofilms. Type III secretion systems (T3SS) are protein-delivery machines which operate by the aid of a sophisticated needle complex to secrete proteins across the bacterial envelope and ultimately deliver them into eukaryotic cells (Galán et al., 2014). Type IV systems use a pilus-based system to transfer DNA or proteins into recipient cells (Cascales and Christie, 2003; Chandran, 2013). The contact-dependent growth inhibition system (CDI) is a Type V system, using a long β-helical cell surface protein to bind to receptors in target cells and deliver a growth inhibitory signal (Hayes et al., 2014; Ruhe et al., 2013). Type VI systems utilize a bacteriophage-like apparatus to puncture the cell envelope to translocate protein effectors into both eukaryotic and prokaryotic target cells (Silverman et al., 2012)

1.5 Pseudomonas putida

Pseudomonas putida strains are frequently isolated from the rhizosphere of plants and many strains promote plant-growth, exhibit antagonistic activities against plant pathogens and have the capacity to degrade pollutants (Arevalo-Ferro et al., 2005; Nelson et al., 2002). Genome comparison of *P. putida* with the human pathogen *P. aeruginosa* PAO1 (Stover et al., 2000) and the plant-pathogen *P. syringae* (Buell et al., 2003) revealed that while there is a high level of genome conservation with the pathogenic species (85% of the predicted coding regions are shared) (Nelson et al., 2002), no genes encoding virulence factors (e.g. type III

protein secretion system (T3SS), exotoxin A, alkaline protease, elastase, a rhamnolipid biosynthesis operon, exolipase or phospholipase C, or plant cell wall-degrading enzymes found in plant pathogens) could be identified in *P. putida* KT2440, being in full agreement with the non-pathogenic lifestyle of this organism (Silby et al., 2011; Wu et al., 2011).

Comparative genomic analysis of four *P. putida* strains (KT2440, W619, F1 and GB-1) showed that the *P. putida* genome is a mixture of some conserved genomic regions and accessory genetic material scattered over the entire genome (Wu et al., 2011). The conserved regions constitute the core genome, which is defined as the genes that are present in all *P. putida* strains. In contrast, the accessory genome includes genes that are found only in some strains, where they tend to cluster in genomic islands (GEIs). The accessory genes have been acquired by horizontal transfer of mobile genetic elements (MGEs) such as phages, plasmids, transposons and integrative and conjugative elements (ICEs) (Hacker et al., 2004). GEIs are are thought to be the major drivers of diversification and adaptation of microorganisms (Juhas et al., 2009). In line with this, it has been shown that many of the accessory capabilities of *P. putida* species , including heavy metal resistance, aromatic compound degradation and stress responses, are encoded on GEIs (Wu et al., 2011).

1.5.1 Biofilms formation in *P. putida*

Several factors have shown to affect biofilm formation and development in *P. putida.* In Figure 4 the most relevant functions are summarized.

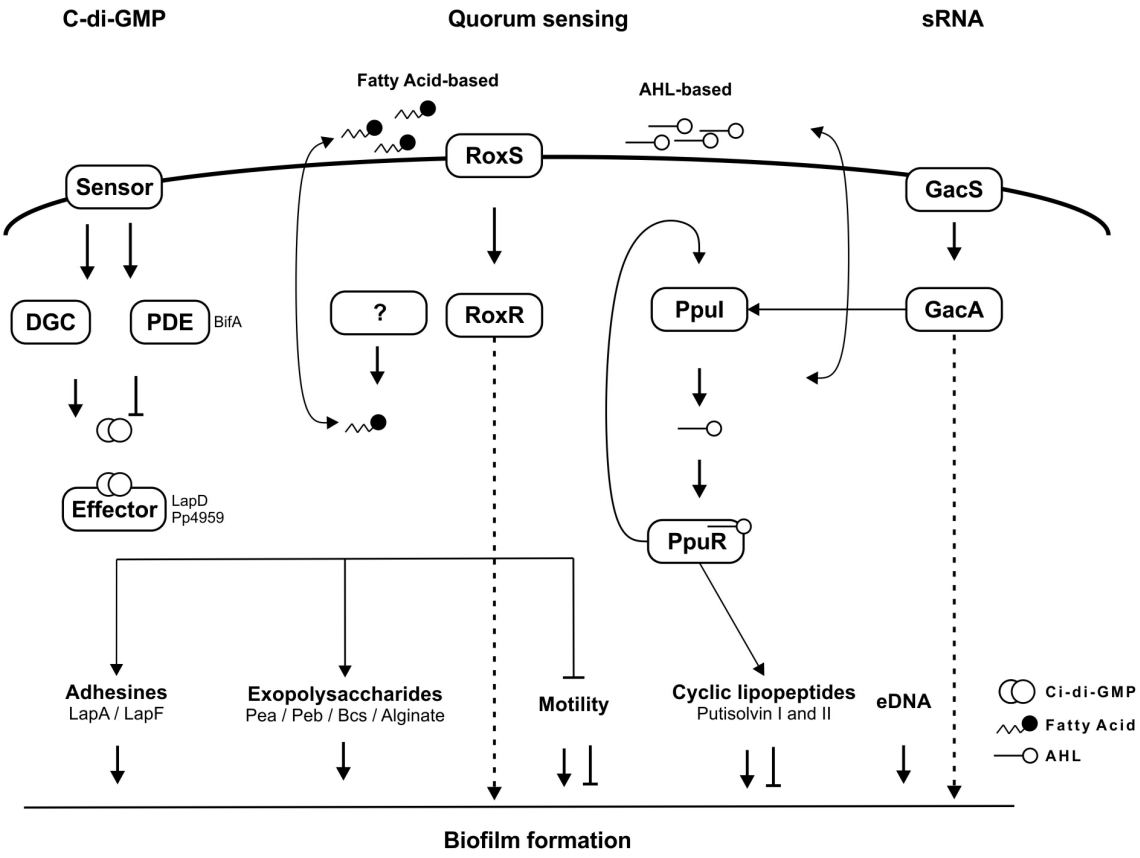

Figure 4: Schematic overview of the regulation of biofilm formation in *P. putida*.

Modified from (Fazli et al., 2014)

1.5.2 Large surface adhesion proteins in *P. putida*

The large surface adhesion protein A, LapA (8682 aa), was found to be required for a stable association with abiotic surfaces and soil particles (Espinosa-Urgel et al., 2000; Gjermansen et al., 2010). A second larges surface protein, LapF (6310 aa), was was shown to be required for cell-to-cell interactions, which are important for cohesion of cells within biofilms (Martínez-Gil et al., 2010). A model has been proposed, in which LapA drives the transition from polar attachment to irreversible surface association, while LapF facilitates cell-to-cell connections within the microcolonies (Fuqua, 2010).

1.5.3 Matrix components and bacterial motility in biofilm formation

P. putida also encodes the biosynthetic machineries for the production of cellulose (*bcs*), alginate, putida exopolysaccharide a (Pea) and putida exopolysaccharide b (Peb). *Pea* contributes to cell–cell interactions and biofilm formation and stability. Bcs plays a minor role in biofilm formation and stability, although it does contribute to rhizosphere colonization as demonstrated by competition assays. In addition, both Pea and Bcs contribute to biofilm formation during hizosphere colonization, and improve the fitness under water-limiting conditions (Nielsen et al., 2011; Nilsson et al., 2011). Flagella driven motility has been shown to be critical for biofilm development after the initial attachment of cells to surfaces (Tolker-Nielsen et al., 2000) and is obviously essential for biofilm dispersal under starvation conditions (Gjermansen et al., 2005).

1.5.4 Quorum sensing and biofilm formation in *P. putida*

AHL-dependent QS systems have been reported in various *Pseudomonas* strains isolated from the rhizosphere (Berg et al., 2002; Elasri et al., 2001; Pierson et al., 2007). The presence of a functional QS machinery in *P. putida* isolates is a strain-dependent trait (Berg et al., 2002; Steindler et al., 2008). In at least tree strains, IsoF (Steidle et al., 2002), PCL1445 (Dubern et al., 2006) and the WCS358 (Bertani and Venturi, 2004), orthologs of an AHL-dependent QS system have been identified. This QS system is located on an genomic island that encodes four proteins: The AHL synthase PpuI, which directs the biosynthesis of 3-oxo-decanoyl-homoserine lactone (3-oxo-C10-HSL) and 3-oxo-dodecanoyl-homoserine lactone (3-oxo-C12-HSL) as major products; the AHL receptor PpuR, which binds to the AHLs and regulates the ppuI expression in a positive feedback loop; RsaL, which is encoded by a gene in the intergenic region between *ppuI* and *ppuR* and has been shown to repress expression of *ppuI* (Rampioni et al., 2012); and the AHL-regulated protein PpuA, which is of unknown function but shows similarity to long chain fatty acid CoA ligases from various organisms. While the wild type *P. putida* IsoF formed very homogenous unstructured biofilms that uniformly cover the surface, a *ppuI* mutant formed structured biofilms with characteristic microcolonies and water filled channels. When the medium was supplemented with AHL signal molecules, the mutant biofilm lost its structure and converted into an unstructured biofilm similar to the one formed by the wild type IsoF. These results suggest that the QS system influences biofilm structural development (Steidle et al., 2002).

1.5.5 Regulation of *P. putida* biofilm formation via c-di-GMP signaling

As for other *Pseudomonas* species (*P. aeruginosa* and *P. fluorescens*), c-di-GMP has been shown as an important regulator of biofilm formation in *P. putida*. It has been demonstrated that the intracellular levels of c-di-GMP modulate the amount of LapA on the cell surface via a regulatory system that involves the periplasmic protease LapG and the inner membrane protein LapD (Fazli et al., 2014). LapG was shown to cleave the N-terminus of LapA thereby releasing it from the cell surface (Gjermansen et al., 2010). The activity of LapG is modulated by the inner membrane protein LapD, which through its periplasmic domain sequesters LapG, preventing it from acting upon LapA (Gjermansen et al., 2005). Additionally, LapD possesses degenerated GGDEF and EAL domains, which bind c-di-GMP. Only when LapD is bound to c-di-GMP, it interacts with LapG. In this way, low levels of c-di-GMP in *P. putida,* result in LapG-dependent dispersal of established biofilms (Gjermansen et al., 2010; Gjermansen et al., 2005; Gjermansen et al., 2006). It has been proposed that a decrease in c-di-GMP levels liberates LapG from LapD, enabling the cleavage and release of LapA from the outer membrane, which eventually leads to a collapse of the structural integrity of the biofilm (Gjermansen et al., 2010). An equivalent mechanism has also been described for *P.* fluorescens (Newell et al., 2011a; Newell et al., 2009; Newell et al., 2011b).

1.6 References Introduction

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The plant-promoting bacterium, *P. putida* IsoF, was isolated (Steidle et al., 2001) and its AHL-based QS system was previously described (Steidle et al., 2002) in our group.

This work was initiated with the idea to monitor the spatiotemporal induction pattern of QS in *P. putida* IsoF biofilms. In the first part of my work I visualized AHL production at the single cell level to examine the link between AHL-based QS, biosurfactant production, and biofilm formation. The second part of my work deals with new insights about the involvement of QS in putisolvin biosynthesis. In the third part of this thesis, I examined the role of contact-dependent growth inhibition of *P. putida* IsoF in bacterial competition within mixed biofilms.

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2 Quorum sensing triggers the stochastic escape of individual cells from Pseudomonas putida biofilms

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Short title: Quorum sensing and non-social behavior

2.1 Abstract

The term ‘quorum sensing’ (QS) is generally used to describe the phenomenon that bacteria release and perceive signal molecules to coordinate cooperative behaviour in response to their population size. QS-based communication has therefore been considered a social trait. Here we show that QS signals (*N*-acyl-homoserine lactones, AHLs) are stochastically produced in young biofilms of *Pseudomonas putida* and act mainly as self-regulatory signals rather than inducing neighbouring cells. We demonstrate that QS induces the expression of putisolvin biosurfactants that are not public goods, thereby triggering asocial motility of induced cells out of microcolonies. Phenotypic heterogeneity is most prominent in the early stages of biofilm development, whereas at later stages behaviour patterns across cells become more synchronized. Our findings broaden our perspective on QS by showing that AHLs can control the expression of asocial (self-directed) traits, and that heterogeneity in QS can serve as a mechanism to drive phenotypic heterogeneity in self-directed behaviour.

2.2 Introduction

The term quorum sensing (QS) is used to describe the phenomenon that bacteria are capable of perceiving and responding to self-generated signal molecules to coordinate their behavior at the group level (Fuqua et al., 1994). The general consensus is that bacteria trigger the QS response only when their cell density has reached a certain threshold (the "quorum"), upon which the expression of target genes is either activated or repressed. Among the various QS signal molecules identified to date, *N*-acyl-homoserine lactones (AHL) have been investigated to the greatest extent (Whitehead et al., 2001; Williams, 2007), and have been shown to control the expression of a large variety of traits, including bioluminescence, virulence, symbiosis, different forms of motility, biofilm formation, production of antibiotics and toxins, and conjugation (Bassler and Losick, 2006; Williams et al., 2007). Many AHL-controlled traits represent cooperative behaviors that can generate benefits to other cells in the local community (West et al., 2007). Consequently, it has been suggested that QS has evolved to restrict the expression of costly cooperative behaviors to conditions, in which they are most beneficial, which is the case at high cell density (Darch et al., 2012; Diggle et al., 2007a; Diggle et al., 2007b; Keller and Surette, 2006; Parsek and Greenberg, 2005; West et al., 2012). However, recent studies in *Pseudomonas aeruginosa* have demonstrated that QS is more complex, because it also controls expression of a few cellular enzymes (i.e. private goods) (Dandekar et al., 2012; Darch et al., 2012; Heurlier et al., 2006; Mellbye and Schuster, 2011). It has been suggested that co-regulation of public and private goods stabilizes cooperation because it negates the selective advantage of cheating mutants, which exploit public goods without contributing to them (Dandekar et al., 2012).

Here, we studied the role of QS in biofilm formation in *P. putida* IsoF, a strain that has been isolated from the rhizosphere of a tomato plant (Steidle et al., 2002; Steidle et al., 2001). In this strain, we have previously identified an AHL-dependent QS system, which is located on a genomic island, encoding PpuI, which directs the biosynthesis of the two AHLs 3-oxo-C10 and 3-oxo-C12 as major products; PpuR, the AHL receptor; and RsaL a repressor of *ppuI* (Steidle et al., 2002). For a closely related strain (PCL1445), it is has been shown that the *ppu* system controls expression of a large nonribosomal peptide synthethase (encoded by *psoA, psoB* and *psoC*), which directs the biosynthesis of the two cyclic lipopeptide biosurfactants putisolvin I and II (Dubern et al., 2006). The putisolvins were found to not only inhibit

biofilm formation of *P. putida* PCL1445 but also to break down existing *Pseudomonas aeruginosa* biofilms (Kuiper et al., 2004). Previous work has shown that *P. putida* IsoF forms a flat and homogenous biofilm, whereas a *ppuI* mutant forms a structured biofilm with characteristic microcolonies and water-filled channel (Steidle et al., 2002). Although putisolvin production has not been demonstrated for *P. putida* IsoF, it has been hypothesized that QS-dependent expression of these biosurfactants could also affect biofilm structural development of this strain (Dubern et al., 2008; Dubern et al., 2006).

In this study, we visualize AHL production at the single cell level to clarify the link between AHL-mediated QS, putisolvin production, and biofilm development in *P. putida* IsoF. We show that at early stages of biofilm development, QS induces putisolvin production, which gives rise to a fraction of motile cells that leave the microcolony on their own. This asocial motility is possible because: (a) AHL signal production is stochastically expressed in only a fraction of the cells in young biofilms; (b) AHL production in one cell does not induce AHL production in its neighboring cells; and (c) putisolvins cannot be used by other cells, and therefore do not represent public goods that can be shared among cells. At a later stage of biofilm development, the AHL expression pattern is more compatible with the expected cross-induction of cells within microcolonies, which results in a mass movement of cells and a concomitant collapse of microcolonies, giving rise to an unstructured biofilm.

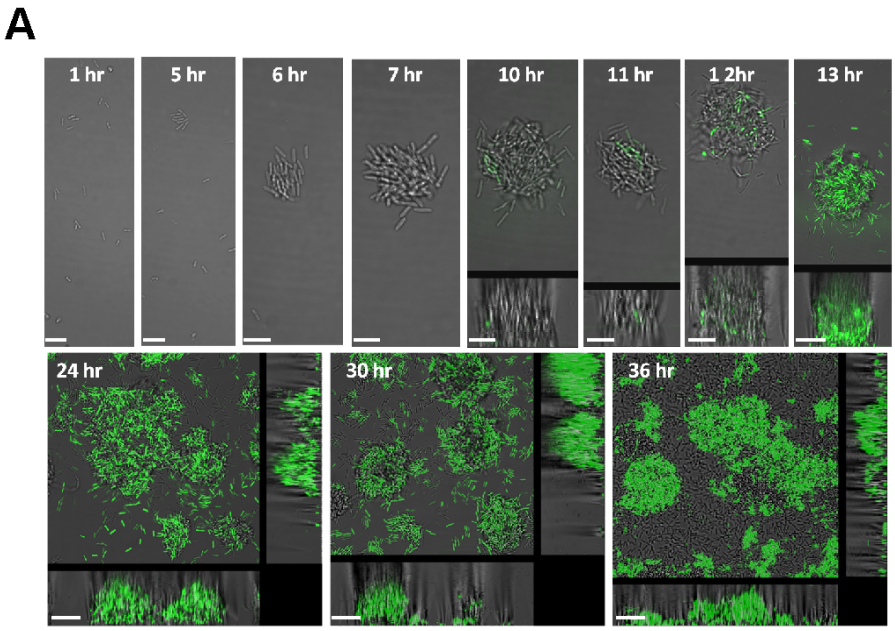
2.3 Results

2.3.1 QS triggers asocial motility in young biofilms of *P. putida*

To investigate the role of AHL-mediated QS in biofilm formation of *P. putida* IsoF at the single cell level we transferred the GFP-based AHL sensor plasmid pRP4las (with stringently controlled copy number: 2-3 per cell) into the wild-type strain. To ensure that none of the cells is AHL-induced prior to inoculation into the flow-through chamber (Lumjiaktase et al., 2010), we grew the strains for at least five generations at a low cell density (OD_{600} < 0.3) in minimal medium supplemented with citrate as carbon source. Following inoculation into the flow-through chambers, we monitored the spatial and temporal production of AHLs during biofilm development under defined conditions (Christensen et al., 1999). In our setup, small microcolonies were formed within 6-8 hours, yet, at this point no fluorescent cells were detectable. When microcolonies reached a population size of 23.3 ± 18.6 bacteria, after 11.9 ± 1.3 hours, a small fraction of cells turned green fluorescent, indicating that they had triggered the production of AHL signal molecules (Fig. 5A). Surprisingly, however, these induced cells did not seem to stimulate AHL production in neighboring cells within the colony as one would expect according to the generally accepted paradigm that QS is a regulatory mechanism that co-ordinates behavior at the group level. To rule out artifacts potentially associated with the use of a plasmid-based AHL reporter we integrated the AHL reporter cassette into the chromosome of *P. putida* IsoF. Using this single copy AHL biosensor we quantified the number of induced cells within and outside of microcolonies. This analysis revealed that free cells outside colonies were significantly more often induced than cells within colonies (Fig. 5B; linear mixed model (LMM): t_{111} = 5.68, P < 0.0001). While the frequency of induced cells significantly increased over time (LMM: t_{111} = 11.73, P < 0.0001), it increased similarly among free and colony cells (LMM, no significant interaction between time and cell status (free vs. colony): t_{111} = 1.14, P = 0.26), showing that QS induction level was consistently higher among free cells. This pattern is compatible with a two-step non-coordinated process starting with stochastic expression of AHL, followed by induced cells becoming motile and independently leaving the microcolonies. Indeed, we observed that induced cells left the colonies and were either removed by the nutrient flow or re-attached to the glass surface in the void spaces between the microcolonies (Fig. 5C).

To further elucidate the heterogeneity in QS induction, we exposed early stage biofilms to a saturating concentration of 3-oxo-C10-HSL (0.5 µM). We observed that the timing of QS induction was slightly advanced (1.5 hours) but that the heterogeneity in induction remained (Supplementary Fig. 1). This supports the idea that there are two distinct subpopulations of QS-responsive and non-responsive cells, whereby cross-induction between the two subpopulations is limited, at least in early stage biofilms. Our observation is reminiscent of previous findings by Pradhan and Chatterjee (Pradhan and Chatterjee, 2014), who demonstrated the presence of stable subpopulations of QS-responsive and non-responsive cells in *Pseudomonas syringae* and *Xanthomonas campestris*.

At later stages of biofilm growth (Fig. 5A), we noticed an increase of fluorescent cell clusters within the microcolonies, which might be the result of AHL-mediated cross-stimulation. These clusters continuously increased in size until the large majority of cells of the microcolonies showed green fluorescence (Fig. 5A; usually in >30 h old biofilms). At this point the microcolonies suddenly collapsed as a consequence of a mass movement of cells. The resulting biofilm was unstructured and uniformly covered the glass surface as has been reported previously for mature IsoF biofilms (Steidle et al., 2002).


A
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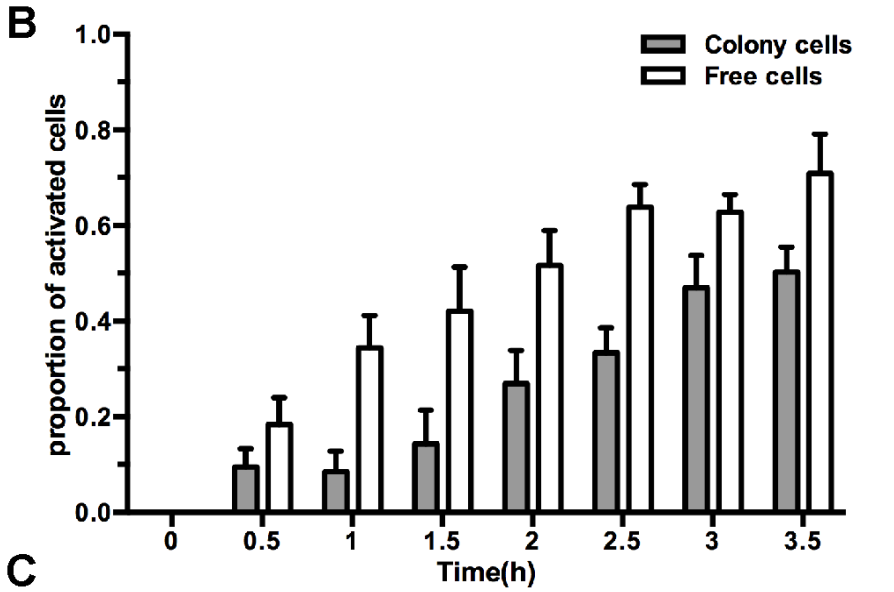
B
Colony cells
Free cells
proportion of activated cells
1.0
0.8
0.6
0.4
0.2
0.0
0
0.5
1
1.5
2
2.5
3
3.5
Time(h)

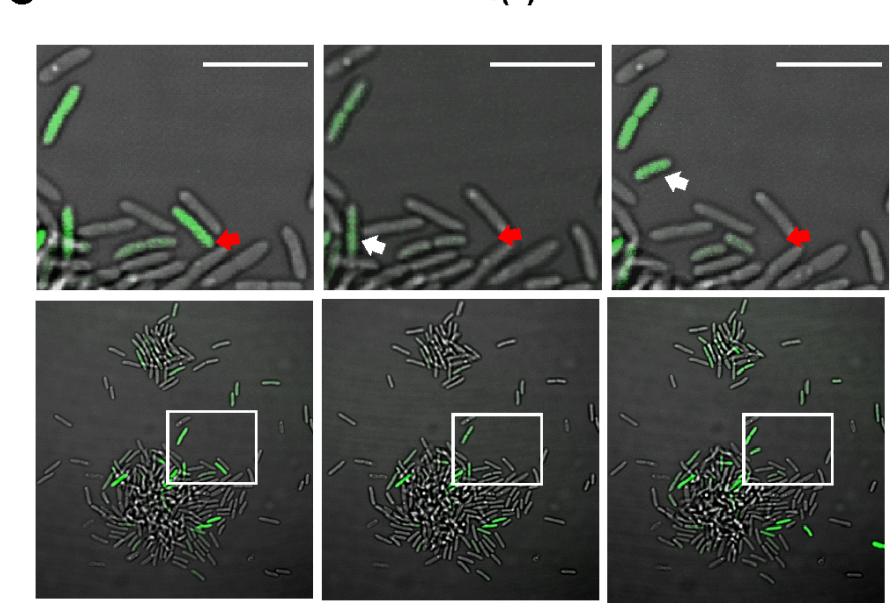
C

Figure 5. AHL production during biofilm development of *P. putida* IsoF.

(*A*) Flow cells were inoculated with a low-density culture of *P. putida* IsoF carrying the GFP-based AHL sensor plasmid pRP4las. Green fluorescence, which is indicative of AHL production, and biofilm formation was followed by CLSM. While AHL expression was stochastic at early stages of biofilm formation, the expression pattern became more homogenous across cells in older biofilms. The large frames show the top view, whereas the right and lower frames show vertical sections through the biofilms. Scale bars: 1-13 h, 5 µm; 24-36 h, 20 µm. (*B*) Focusing on early stages of biofilm formation (between 8 and 14 h post inoculation), quantitative analysis from 8 independent frames revealed that the proportion of AHL-activated cells was significantly higher among free cells than among cells within colonies. This pattern remained consistent over time. Time 0 was defined as 30 min before the first appearance of induced cells (mean: 10.5±0.8 h post inoculation). (*C*) Many of the AHL-induced cells became highly motile und moved out of the microcolony, as exemplified in pictures from left to right, which were taken at 30 min intervals. Upper panel shows a close-up of the region indicated in the lower panel. The red arrow points at a cell that left the microcolony and the white arrow indicates a cell that moved to the periphery of the microcolony. Scale bar: 5 µm.

2.3.2 Putisolvin is required for motility and biofilm collapse

In a next step, we aimed to better understand the mechanistic link between QS-heterogeneity and asocial motility in our system. Because previous work had revealed a strong relationship between the production of putisolvin biosurfactants and biofilm formation in *P. putida* PCL1445 (Dubern et al., 2006; Kuiper et al., 2004), we examined whether strain IsoF also harbors the putisolvin biosynthetic gene cluster, and whether asocial motility and biofilm collapse is linked to putisolvin production. By using a PCR approach combined with sequencing, we found that the entire *pso* gene cluster is present in strain IsoF and shows more than 99% DNA sequence identity with the *pso* locus of strain PCL1445. To investigate whether putisolvins act as a biosurfactant in the IsoF strain, we constructed the defined *psoA* knock-out mutant PL11, as well as the conditional mutant PL2, in which the native promoter region of *psoA* has been replaced with the rhamnose-inducible P_{rhaB}

promoter (Supplementary Fig. 2). In the absence of rhamnose, both mutant strains showed no surfactant activity in a simple drop collapse assay (Fig. 6A). However, drop collapsing activity of strain PL2 but not of PL11 could be restored when the medium was amended with at least 0.5% rhamnose. The Du Nouy ring method was used to measure surface tension of spent culture supernatants along the growth curve. Surface tension was found to reach a minimum when the cultures had an OD_{600} of approximately 2.0. Importantly, surface tension was found to be significantly reduced upon rhamnose-induced putisolvin production (Fig. 6B). Next, we tested whether putisolvin is essential for swarming motility, as has been found to be the case for other biosurfactants in other bacterial species (Kearns, 2010). Indeed, when tested on swarming plates containing citrate as carbon source, the wild-type IsoF colonized the entire plate within 3 days whereas no surface migration was observed for mutants PL2 and PL11 (Fig. 6C). However, in the case of PL2 swarming could be restored by amending the medium with 0.5% rhamnose (Fig. 6D), whereby the migration speed of the swarm colony was found to be proportional to the rhamnose concentration. These results demonstrate that putisolvin acts as a biosurfactant in *P. putida* IsoF, and is essential for swarming.

To test whether putisolvin is involved in biofilm collapse, we compared biofilm formation of the wild-type IsoF to the conditional *psoA* mutant PL2 in flow-through cells using AB minimal medium supplemented with 1 mM citrate. After 3 days of growth the wild-type had formed a flat and unstructured biofilm with a low volume/area ratio, while the biofilm of mutant PL2 was dominated by large microcolonies, characterized by a three-fold higher volume/area ratio, and with only few cells colonizing the void space (Fig. 7). Addition of 0.2% rhamnose to the medium recovered the flat wild-type biofilm structure. These experiments demonstrate that putisolvins promote the colonization of the substratum by facilitating the movement of cells out of microcolonies.

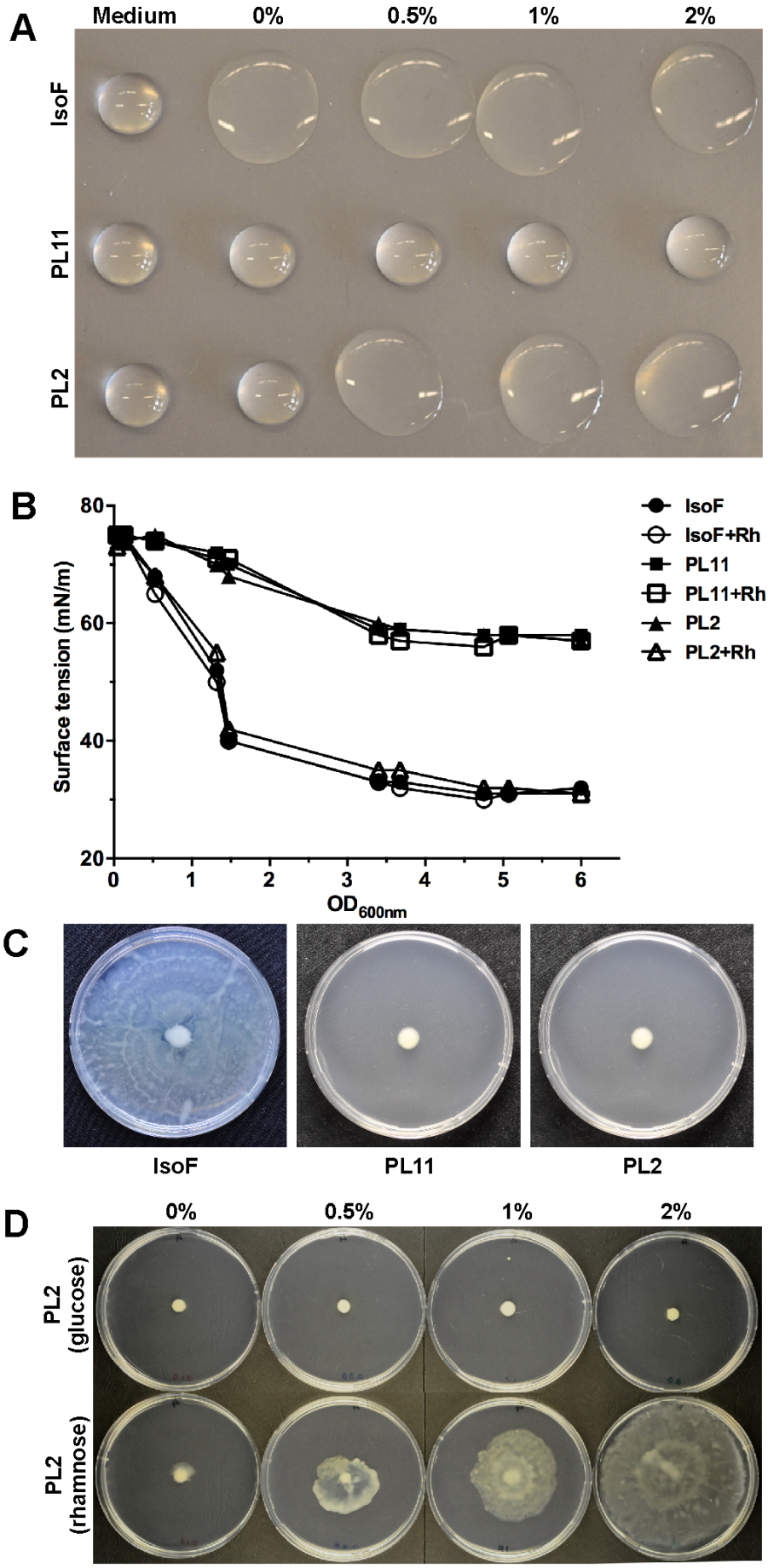
A
Medium
0%
0.5%
1%
2%
IsoF
PL11
PL2
B
Surface tension (mN/m)
80
60
40
20
0
1
2
3
4
5
6
OD600nm
IsoF
IsoF+Rh
PL11
PL11+Rh
PL2
PL2+Rh
C
IsoF
PL11
PL2
D
0%
0.5%
1%
2%
PL2 (glucose)
PL2 (rhamnose)

Figure 6. The production of biosurfactants and swarming motility in *P. putida* IsoF are dependent on the *pso* gene cluster.

The wild type IsoF, the *psoA* mutant PL11 and the conditional *psoA* mutant PL2 were grown in the absence or presence of rhamnose. (*A*) In drop collapse assays, surface tension of supernatants of overnight cultures were found to be increased in PL11 compared to IsoF, but could be restored to wild-type level in PL2 upon rhamnose supplementation. (*B*) Surface tensions of PL2, PL11 and IsoF in medium containing 1% or no rhamnose were quantified along the growth curve using the Du Nouy ring method. Results are representative of three independent experiments. (*C*) IsoF displays swarming motility in ABC medium, whereas PL11 and PL2 are impaired in swarming because they do not produce putisolvin. *(D)* Swarming of the conditional *psoA* mutant PL2 was abolished on glucose plates, but was increasingly restored on plates supplemented with 0.5%, 1% or 2% rhamnose. Pictures were taken after 3 days of incubation.

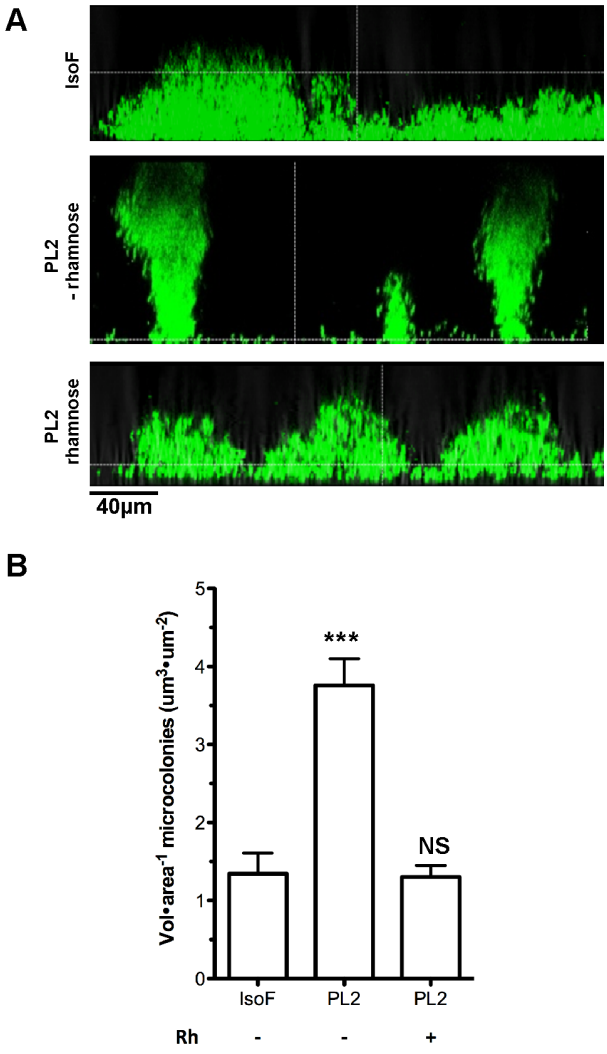

Figure 7. The role of putisolvin biosurfactants in biofilm structural development.

Flow chambers were inoculated with *gfp*-tagged derivatives of the wild type IsoF and the conditional *psoA* mutant PL2 in the absence or presence of 0.2% rhamnose. *(A)* Vertical sections through the biofilms after three days show a flat biofilm structure for IsoF and PL2 amended with rhamnose, whereas PL2 without rhamnose forms biofilms that are dominated by towering microcolonies. *(B)* 3D parameter analysis of biofilm structures show that microcolonies of IsoF have significantly lower volume/area ratio than microcolonies of PL2. The wild type phenotype could be restored in PL2 when adding rhamnose (Rh) to the medium. Mean values of three independent experiments are shown with s.e.m. (one-way ANOVA; *** $p<0.001$; NS, not significant).

2.3.3 Putisolvin production is QS-regulated

We further investigated whether AHL-mediated QS controls putisolvin production, thereby leading to the asocial motility phenotype and biofilm collapse observed in our single-cell experiments. Indeed, Dubern *et al*. have demonstrated that production of putisolvins in strain PCL1445 is regulated by the *ppuI-rsaL-ppuR* QS system (Dubern et al., 2006). In agreement with this study, we found that a *ppuI* mutant of strain IsoF, named F117, was unable to swarm, and spent culture supernatants of F117 cultures lacked surfactant activity (Fig. 8A and 8B). Both defects could be rescued by the addition of 5 µM 3-oxo-C10-AHL. These results strongly suggest that QS-deficiency in F117 results in the abolishment of putisolvin production. To obtain more direct evidence for the link between QS and putisolvin production, we constructed a transcriptional fusion of the *psoA* promoter (triggering putisolvin synthesis) with *gfp* and transferred the resulting plasmid, pLUM1, into the wild-type IsoF, the *ppuI* mutant F117 (defective for AHL signal production), and the *ppuR* mutant GC3 (defective for responding to AHL signals). Measurements of GFP fluorescence revealed that *psoA* expression was strongly decreased in F117 and GC3, but could be restored to wild-type level for F117, but not for GC3, when the medium was supplemented with 5 µM 3-oxo-C10-HSL (Fig. 8C).

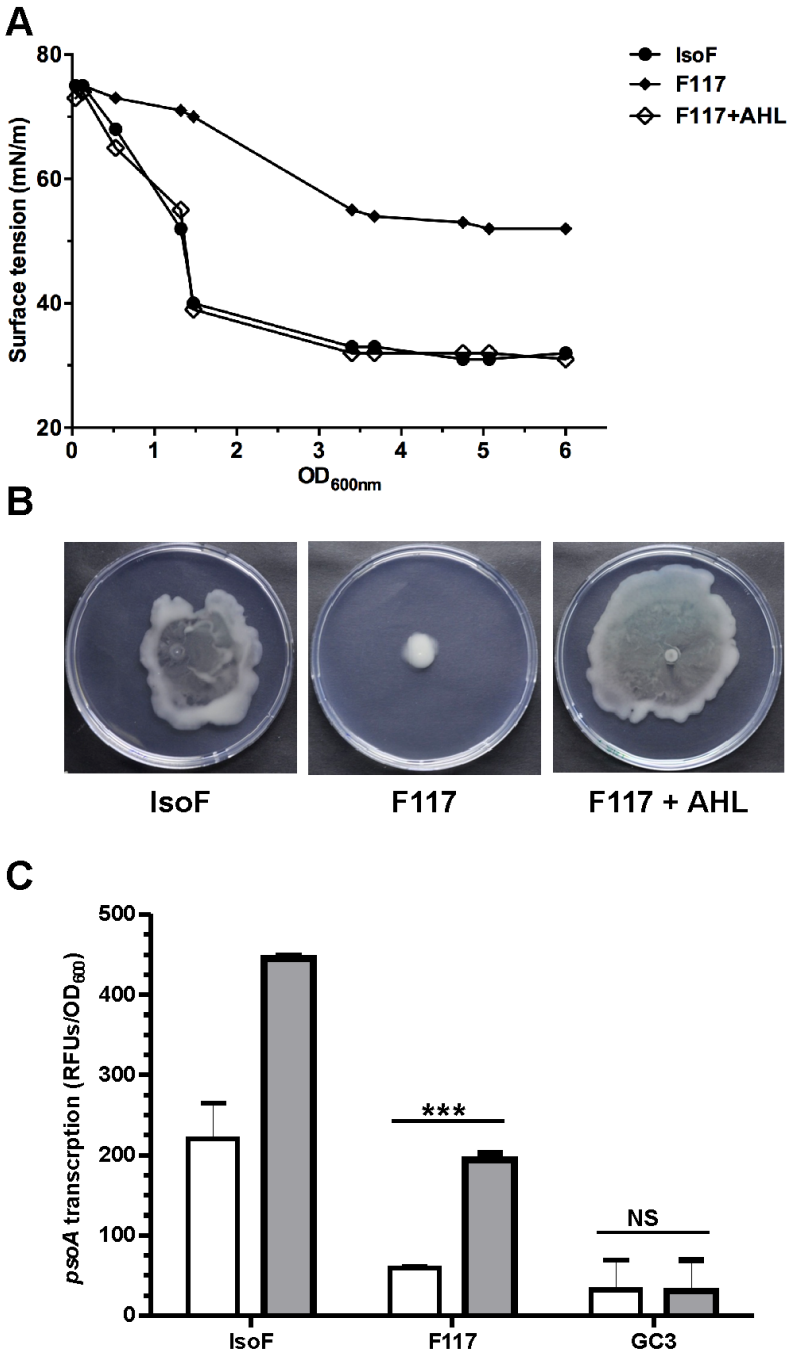

Figure 8. Putisolvin production and swarming motility are controlled by the *ppu* QS system in *P. putida* IsoF.

The surface tension (*A*) and swarming motility (*B*) of the *ppuI* mutant F117 increased and decreased, respectively, but were restored to wild-type IsoF levels upon supplementation with 5 µM of 3-oxo-C10-AHL. Surface tension of supernatants was measured by the Du Nouy ring method. Results are representative of three independent experiments. Swarming plates were photographed after 3 days of incubation. (*C*) When measuring the *psoA* promoter activity in ABC medium in the absence (open bars) or presence of 5 µM of 3-oxo-C10-AHL (gray bars), *psoA*

promoter activity could be restored to wild-type IsoF level in the *ppuI* mutant F117 (which does not produce AHL signals), but not in the *ppuR* mutant GC3 (which cannot respond to AHL signals). Mean values of three independent experiments are shown with s.d. (one-way ANOVA; *** $p<0.001$; NS, not significant).

2.3.4 The role of flagella for biofilm development of *P. putida*

To investigate if, in addition to putisolvins, flagella may be required for cell migration out of microcolonies we constructed a *fliM* mutant of *P. putida* IsoF, designated GC25, which no longer produces flagella and therefore is unable to swim. The microcolonies that strain GC25 formed were much more compact than the ones of the wild-type strain, suggesting that flagella are important for the positioning of cells within the aggregates (Fig. 9). Moreover, putisolvin producers migrated only at a very low rate out of the microcolonies when compared to the wild-type. These results show that the dissociation of cells from microcolonies is dependent on both flagella-driven motility and the production of putisolvin biosurfactants.

2.3.5 Spatial expression of *psoA* in biofilms

We followed the temporal and spatial expression of putisolvins within biofilms of *P. putida* IsoF using the P_{psoA}-*gfp* transcriptional fusion. Similar to our previous results (Fig. 1), we found that fluorescent cells were mainly located at the periphery or outside of microcolonies. In contrast, when the same transcriptional fusion was present in the putisolvin-defective mutant PL11 or the non-motile mutant GC25, we observed both a higher proportion of QS-induced cells and a more homogenous induction across cells, especially in GC25 (Fig. 9). These findings provide evidence that asocial cell movement out of the colony restricts cross-induction in the wild-type strain, while cross-induction becomes increasingly possible when cells are forced to stay together.

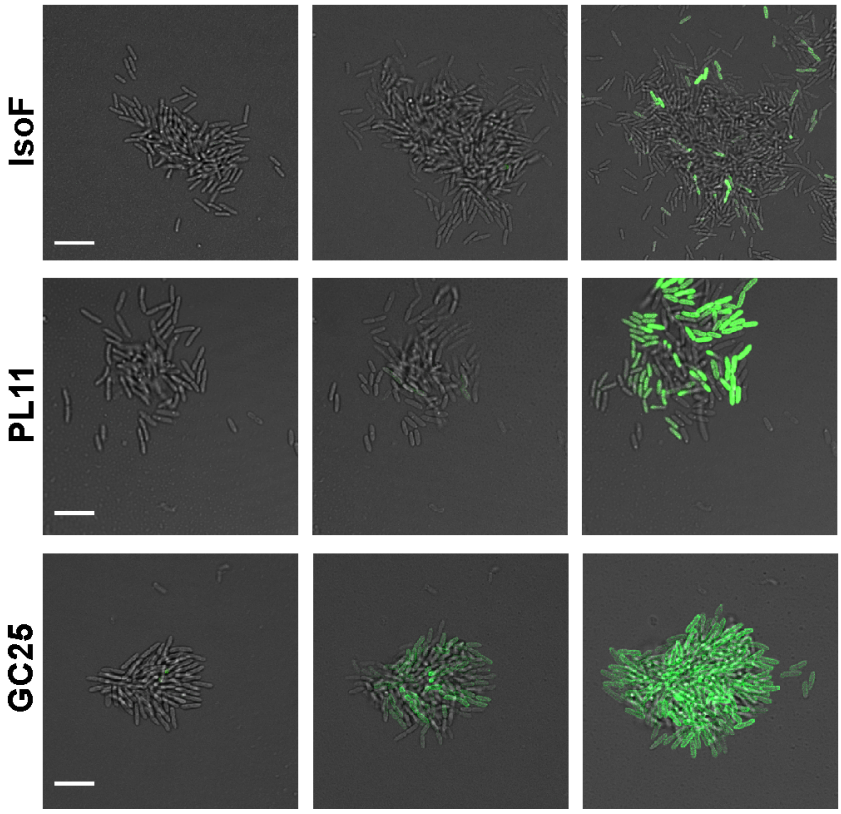

Figure 9. Activity of the *psoA* promoter during biofilm development.

Early stage biofilm development of the wild type IsoF, the *psoA* mutant PL11 and the non-motile strain GC25 was followed over time. Strains were transformed with plasmid pLUM3a, carrying a *psoA-gfp* transcriptional fusion. The activity of the *psoA* promoter was visualized by fluorescence microscopy at 30 min intervals. Due to impaired motility of the strains, microcolonies of strains PL11 and GC25 were more compact, and contained a higher proportion of fluorescent cells than wild-type colonies. Scale bar: 5 µm.

2.3.6 Putisolvins are private and not public goods.

A recent study in *P. aeruginosa* revealed that biosurfactants can represent public goods, which allow biosurfactant-defective mutants to swarm along with biosurfactant-producing wild-type cells (Xavier et al., 2011). In contrast, our single-cell analyses suggest that biosurfactants in *P. putida* IsoF remain associated with the bacterial cell surface, thereby triggering individual-based and not group-based swarming. To test this hypothesis, we mixed a putisolvin-deficient or a flagella-deficient mutant with the wild-type on swarming plates. In support of our hypothesis, we found that the mutants stayed close to the inoculation point, forming a small colony, whereas the wild-type swarmed over the entire plate (Fig. 10), with swarming behavior being unaffected by the presence of another strain (Supplementary Fig. 3). This result held across a wide range of strain mixing ratios (Supplementary Fig. 3 and Fig. 4). These findings demonstrate that putisolvins are private rather than public goods, which mostly adhere to the producing cells, and can therefore not be used by other cells for swarming.

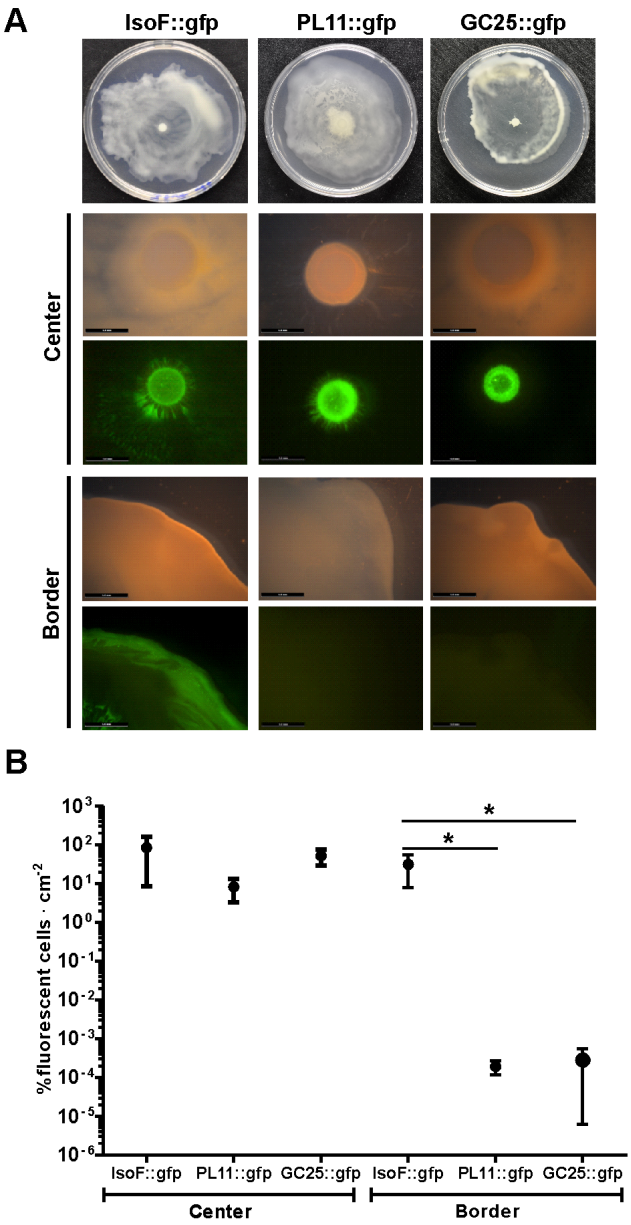

Figure 10. Putisolvins are not a public good in swarming colonies.

Swarming plates were inoculated with equal amounts of the unmarked wild type strain IsoF and GFP-marked derivatives of either IsoF, the putisolvin-deficient mutant PL-11 or the non-motile strain GC25. (*A*) Microscopic inspection revealed that the GFP-marked wild type strain was present at the border of the swarm, while strains PL-11 and GC25 were mainly restricted to a region close to the inoculation point. Scale bar: 5 mm. (*B*) The dissemination of cells within the swarm was quantified by plating cells taken from the center or the border region of the swarm on selective medium. Mean values of three independent experiments are shown with s.d. (One-way ANOVA;* $p<0.05$).

2.4 Discussion

Our work breaks with the central assumption that QS primarily represents a regulatory mechanism to coordinate cooperative behaviors among cells at high population density. Instead, we show that QS can also do the opposite: trigger uncoordinated self-directed behavior at low cell density. Specifically, we found that in the early stages of biofilm development AHL production occurred stochastically in only a fraction of cells. AHL production in these cells triggered the synthesis of putisolvins, biosurfactants that remain associated with the producer's cell surface, thereby resulting in cells individually moving out of the microcolony. Since this asocial motility removes individuals with the highest AHL production from the consortium, it exerts a negative feedback on cells left behind by delaying AHL cross-induction within the microcolony. It is important to note that we were only able to discover the lack of cross-induction and asocial motility because AHLs were stochastically expressed at low population density. If all cells had started expressing AHLs at the same time, we would have erroneously concluded that putisolvin serves as a public good, coordinately expressed in the consortium to allow cooperative motility. This highlights that the mere observation of individual cells doing the same thing at the same time does not necessarily mean that coordination through communication and the sharing of pubic goods are involved (Ross-Gillespie and Kummerli, 2014).

There are at least three reasons why stochasticity can arise in our system. First, Kaplan and Greenberg (Kaplan and Greenberg, 1985) showed that AHL-dependent QS can be an extremely sensitive system, as demonstrated in *Vibrio fischeri*, where one to two AHL molecules per cell are sufficient to trigger autoinduction, suggesting that at very low AHL concentrations QS is intrinsically stochastic. Second, although the classic QS model assumes that AHLs are diffusing into the cell from the surroundings, such that the population density determines induction levels, we suggest that at the onset of QS the signal molecules are not released from the producing bacterium but directly bind to their cognate cytoplasmic receptors, which, as a consequence, results in self-induction of the cell's QS cascade, and not in cross-induction. This possibility is especially probable in our study system, as the *P. putida* IsoF AHLs contain relatively long fatty acid chains, which often require transporters to be actively released from the cell (Buroni et al., 2009; Chan et al., 2007; Pearson et al., 1999). Third, physiological differences between cells, particularly when grown as a biofilm, may exist that affect AHL

production or the sensitivity of the QS response. Moreover, cross-induction seems to be additionally impeded in our system because induced cells leave the consortium, which presumably results in reduced local AHL concentrations.

While previous work has revealed heterogeneity in QS both at low and high cell densities, the situation described in this study is unique, as it is the first example that QS heterogeneity serves as a mechanism to trigger a self-directed behavior of individual cells. At low cell density, heterogeneity in the initiation of QS has been observed in *P. aeruginosa* when single cells were confined in small volumes in a microfluidic device (Boedicker et al., 2009). In this study it is not only shown that QS induction is highly variable but also that low numbers of cells, even single cells, are able to initiate QS, supporting the idea of QS self-induction. At high cell density, meanwhile, Anetzberger *et al.* (Anetzberger et al., 2009) showed that the expression of QS-regulated bioluminescence in *Vibrio harveyi* is heterogenous. In a subsequent study a working model was presented, in which the combination of the different *V. harveyi* signal molecules available (this organism produces three structurally unrelated signal molecules), rather than cell density *per se*, determines the timing of QS-regulated traits in this species (Anetzberger et al., 2012). Likewise, induction of AHL-controlled bioluminescence in individual *Vibrio fischeri* cells was found to differ widely in time scale and in the overall intensity, suggesting that QS has relatively imprecise control over the response of an individual cell (Perez and Hagen, 2010). Although heterogeneity in QS seems frequent, a remaining key question is whether the heterogeneity is simply an inevitable outcome of the regulatory mechanism controlling AHL production, or whether it has an adaptive function. While speculative at this stage, it seems plausible that, in our case of asocial motility, leaving the microcolony can be beneficial under nutrient depletion. Even more so in mixed biofilms, where natural selection could favor individual-based early dispersal to reach new resources faster than the competitors (Rendueles and Ghigo, 2012).

While our data indicate that both AHL and putisolvin production is self-directed and thereby asocial during the early stages of biofilm formation, the pattern conceivably changed in older biofilms. Although AHL-producing cells first leave the microcolonies by themselves, we hypothesize that over time AHLs accumulate in the microcolonies, eventually leading to cross-induction of neighboring cells. This idea is supported by the observation that *psoA* expression is more homogenous and increases faster in mutants impaired in motility when compared with expression of *psoA* in the wild-type background (Fig. 5). Furthermore, typical AHL cross-induction

of cells has been observed in *P. putida* IsoF microcolonies grown in a microfluidic setup, in which motility of cells was restricted by coating the substratum with polylysin (Meyer et al., 2012). Thus, at later stages of biofilm development AHL signaling eventually becomes a social trait, which then results in all cells producing putisolvins. This, in turn, leads to the hallmark of biofilm structural development in our setup, which was the sudden collapse of microcolonies at high cell densities. Because nutrients are likely limiting at this point of biofilm development, even self-directed motility can become a social trait because putisolvin-mediated biofilm dispersal will prevent overcrowding and allow the left behind population to resume growth. Indeed, the putisolvin-mediated microcolony collapse is reminiscent to the role of QS-controlled rhamnolipid production in detachment of cells from mature *P. aeruginosa* biofilms, which was suggested to release the stress arising from nutrient limitation at high population density(Boles et al., 2005).

Our experiment showing that putisolvin-deficient mutants are unable to swarm with the wild-type strain demonstrates that putisolvins do not represent public goods (Fig. 6). In support of this, it has been shown that putisolvins preferentially adhere to the cell surface of the producing cell (Kuiper et al., 2004). Interestingly, adhesion to bacterial cell surfaces has also been shown for other biosurfactants (Neu, 1996; Yuan et al., 2007; Zhong et al., 2007), which may therefore represent a more general phenomenon. In analogy to our findings, Burch *et al*. showed that the biosurfactant syringafactin, which is produced by the plant epiphyte *Pseudomonas syringae* pv. syringae B728a, is adsorbed to the adjacent waxy cuticle or retained on the bacterial cell surface (Burch et al., 2014). It is shown that its production primarily benefits the producer both by attracting moisture and facilitating access to nutrients.

Our results relate to recent work on QS in *P. aeruginosa*, where it has been shown that QS not only coordinates the expression of public goods at the group level, but also directs the expression of metabolically important enzymes at the cellular level (Dandekar et al., 2012; Heurlier et al., 2006). The QS regulatory control over both social and self-directed traits has been interpreted as an adaptation to prevent invasion of cheating mutants. The idea is that the fitness increase a QS-deficient mutant gains by exploiting a QS wild-type strain, is cancelled by the fitness loss these mutants face because they lack an important cellular enzyme. The situation is clearly different in our study system where the risk of cheating is reduced because both the AHL-signal and putisolvins are not or only partially available to others. Taken together, our insights highlight that QS is much more complex than

previously thought, as the traits being induced by QS can cover the entire continuum from a cooperative public good trait that generates benefits to others (e.g. elastase production (Diggle et al., 2007b)), to extra-cellular traits that mostly generate self-directed benefits (e.g. putisolvin production), to entirely intra-cellular traits that solely provide benefits to the producer.

2.5 Methods

2.5.1 Strains and culture conditions

Bacterial strains and plasmids used in this study are listed in Supplementary Table S1. *Escherichia coli* strains used for recombinant manipulations were propagated in Luria-Bertani (LB) medium at 37°C. Plasmids were delivered to *P. putida* by triparental mating (Christensen et al., 1999). Briefly, donor, recipient and helper strain, *E. coli* HB101(pRK600), were harvested from overnight cultures, mixed and spot-inoculated on LB plates. After overnight incubation at 37°C transconjugants were isolated on Pseudomonas Isolation Agar (PIA) at 30°C. *P. putida* strains were grown in modified AB medium supplemented with 10 mM sodium citrate(Heydorn et al., 2000) (referred to as ABC medium). When required, media were supplemented with antibiotics at the following concentrations. For *E. coli*: 50 µg ml^{-1} ampicilin, 50 µg ml^{-1} kanamycin, 10 µg ml^{-1} gentamycin, 10 µg ml^{-1} tetracycline, 50 µg ml^{-1} trimethoprim. For *P. putida*: 100 µg ml^{-1} kanamycin, 20 µg ml^{-1} gentamycin, and 100 µg ml^{-1} tetracycline.

2.5.2 Construction of *P. putida* IsoF mutants

The *psoA* mutant PL11 was generated as follows: an internal *psoA* fragment was PCR amplified using the primers psoAF (5'-ctgatggtgtcgttcgaagagg-3') and psoAR (5'-gctcgtcgagcacgtacaactg-3'). The amplicon was digested with SmaI and cloned into the gene replacement vector pEX18Gm cut with the same enzyme. The resulting plasmid, pEX18*psoA*, was mobilized into *P. putida* IsoF by triparental mating, and gene replacement mutants were selected on PIA medium containing 20 µg ml^{-1} gentamycin. The *fliM* mutant GC25 was constructed by amplifying a *fliM* internal fragment using primers FliMF (5'-gccatggccgggttgaytc-3') and FliMR (5'-gaygaygggctggtrcagac-3') and cloning the PCR product blunt-ended into the StuI-digested gene replacement vector pSHAFT2Gm. The resulting plasmid, pSHAFT2*fliM*, was used to construct a *fliM* mutant as described for the *psoA* mutant. A *pso* conditional mutant was constructed as follows: first the gentamycin resistance cassette from pBBR1MCS-5 was amplified using the primers genF (5'-gcagcaacgatgttacgcag-3') and genR (5'-ttggtaccccgatctcggcttgaacg-3'), the amplicon was digested with XbaI and KpnI (restriction site underlined) and cloned into plasmid pSC200 cut with the same enzymes, yielding plasmid pSC200Gm. Next, a 590-bp fragment beginning at the start codon of *psoA* was amplified using primers pos2F (5'-

tgcctgccgccgaaacctt-3') and pos590R (5'-atctagagccagccaataatcgcggtc-3') and the resulting DNA fragment was blunt ended with Klenow fragment and cloned into the filled-in NdeI site of pSC200Gm. This plasmid was mobilized from *E. coli* CC118 into *P. putida* IsoF by conjugation and the conditional mutant was selected on PIA medium supplemented with 20 µg ml^{-1} gentamycin. The genetic structures of all mutants constructed were confirmed by PCR and sequence analysis. The following primers were used: pSHAFT2F (5'-CGCTCTCGCGGCTTACGTTC-3'), pSHAFT2R (5'- AAGCCAGGGATGTAACGCACTG -3'), peX_F (5'-CACCGACAAACAACAGATAA-3'), peX_R (5'-CCCCAGGCTTTACACTTT-3') pSC200end (5'-GTCATACTGGCCTCCTGATGTCGT-3').

2.5.3 Construction of transcriptional fusions

The pUT/mini-Tn*5*Km-based plasmid pPLlas (Lumjiaktase et al., 2010) was used to integrate the GFP-based AHL sensor into the chromosome of *P. putida* IsoF. Three independent mutants with different insertion positions were purified and used for flow cell experiments. To construct a P_{psoA}-*gfp* transcriptional fusion the psoA promoter region was PCR amplified using the primers p-psoAF (5'-aggatccgattctaagctttgcggcg-3') and p-psoAR (5'-tggatccgctcagggcaaaggtttcg-3'). PCR fragments were cloned as *BamHI* fragments (restriction sites are underlined) into the respective site of the promoter-probe vector pGA-G1, generating the plasmids pPLM1 (P_{psoA}-gfp*). A* P_{psoA}-*cfp* fusion was generated by cloning the PCR product containing the *ecfp* gene from pBK-mini-Tn*7* into the TOPO vector. Then the psoA *promoter region from PLM1* was inserted as a BamHI fragment upstream of the *ecfp* gene in this plasmid. Finally, the the P_{psoA}-*cfp* cassette was excised as a SacI fragment and inserted into the same site of plasmid pBBR1MCS-3, yielding pLUM3 (P_{psoA}-*cfp*). The plasmids were mobilized from *E. coli* CC118 to *P. putida* strains by conjugation and selected on PIA medium supplemented with 50 µg ml^{-1} gentamycin (pPLM1) or 100 µg ml^{-1} tetracycline (pLUM3).

Measurement of promoter activities

P. putida strains harboring pPLM1 were grown in 10 mM ABC medium for 24 hrs at 30°C with continuous shaking. When required, 3-oxo-C10 homoserine lactone was added to the medium at a final concentration of 5 µM. Green fluorescence was measured using 200 µl samples in a microtitre plate reader (SynergyTM HT, MWG Biotech, Germany) with an excitation wavelength of 485 nm and emission detection

at 528 nm. The data were corrected for autofluorescence and processed with the KC4 software (BioTek Instruments). Specific fluorescence was calculated by normalizing relative fluorescence to OD_{600}, which was simultaneously measured in the instrument.

2.5.4 Swarming motility assays

Swarming motility was determined on ABC agar plates supplemented with 0.1% casamino acids and solidified with 0.4% (wt/vol) agar as described previously (Eberl et al., 1996). Briefly, overnight cultures were adjusted to an OD_{600} of 0.1, and 2 µl samples were inoculated on swarming plates, which were incubated for 3 days at 30°C. The swarming plates were supplemented with 0.5%, 1% or 2% (wt/vol) rhamnose when appropriate.

2.5.5 Biosurfactant production

Semi-quantitative measurement of biosurfactant activity was done by using the drop-collapsing assay, in which the reduction of surface tension causes a collapse of the droplet placed on a hydrophobic surface. To quantify biosurfactant production the decrease of surface tension between culture medium and air was determined with a Du Nouy ring (Dubern et al., 2006).

2.5.6 Cultivation of biofilms, microscopy, and image analysis

Biofilms were grown in flow cells supplied with ABC medium. The flow system was assembled and prepared as described previously (Christensen et al., 1999). Briefly, the flow channels were inoculated with *P. putida* cultures grown for at least five generations at a low cell density (OD_{600} < 0.3) in minimal medium supplemented with citrate as carbon source. The medium flow was kept at a constant rate of 0.2 mm s^{-1} by a Watson-Marlow 205S peristaltic pump. The incubation temperature was 30°C. Microscopic inspection and image acquisition were performed using a confocal laser scanning microscope (CLSM) (DM5500Q; Leica) equipped with a x40/1.3 or a x63/1.4 oil objective. Captured images were analyzed with the Leica Application Suite (Mannheim, Germany) and the Imaris software package (Bitplane, Switzerland). Images were prepared for publication using CorelDraw (Corel Corporation) and PowerPoint (Microsoft) software.

To quantify the proportion of AHL-induced and non-induced cells, five independent experiments were conducted. In each experiment, five random positions were chosen on the flow chamber glass surface, and surveyed every 30 minutes for 10 hours, starting 6 hours post inoculation. Aliquots of a low-cell-density inoculum (OD_{600} = 0.01) were used to initiate the flow cell biofilms to allow single cells analysis. To distinguish between free and colony-associated cells, an aggregate size of eight cells was defined as threshold, below which cells were considered as free. Using this threshold, the average aggregate size for free cells was found to be 3.5±0.5. Since transmitted light was used to obtain the total cell number only microcolonies with few cell layers were used for quantification. When analyzing older biofilms consisting of multiple cell layers, we either used strains marked with mCherry or stained cells with SYTO 62 (Life technologies).

2.5.7 Statistical analysis

A linear mixed model (LMM) was used to test whether the proportion of AHL-induced cells differs between free and colony-associated cells, and whether the induction pattern changes over time. Position identity within experiments was introduced into the model as a random factor to account for the nested approach (i.e. five positions within five experiments). Prism (GraphPad Software) was used for one-way ANOVA. If the ANOVA yielded significant differences between factor levels, the Bonferroni method was applied for pairwise comparisons between factor levels.

2.5.8 Acknowledgments

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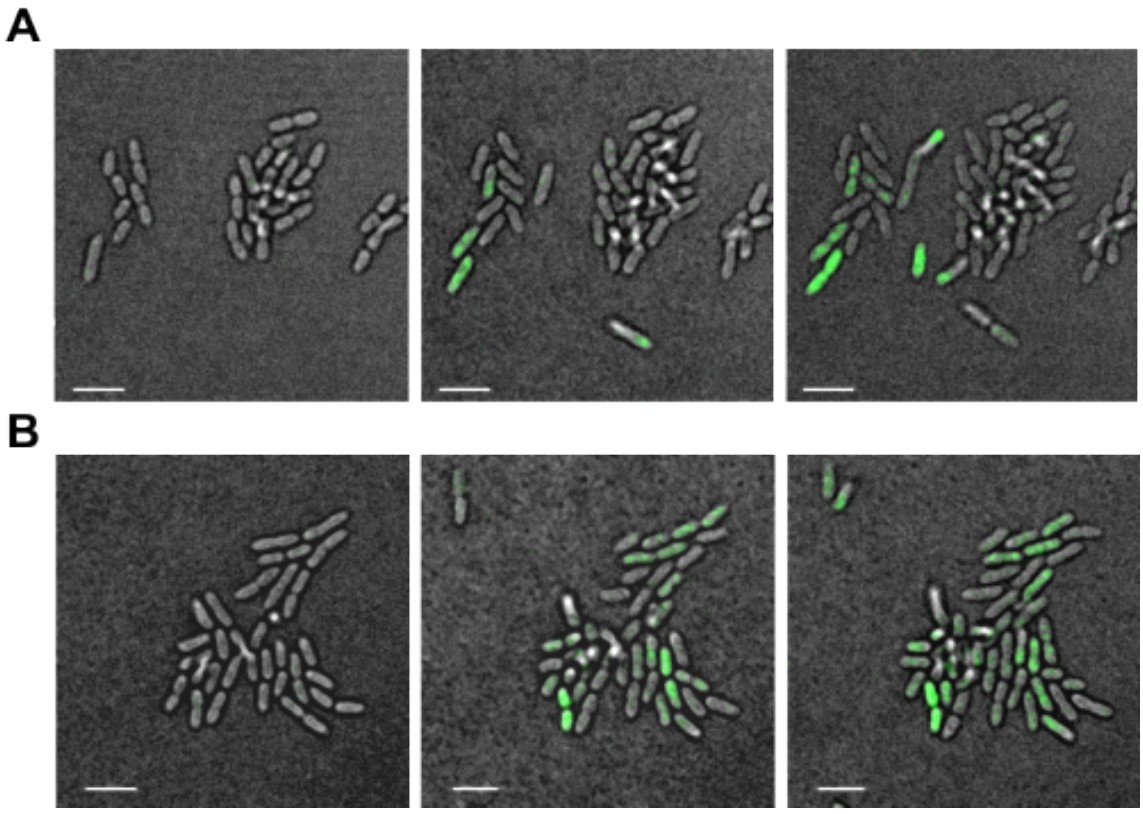
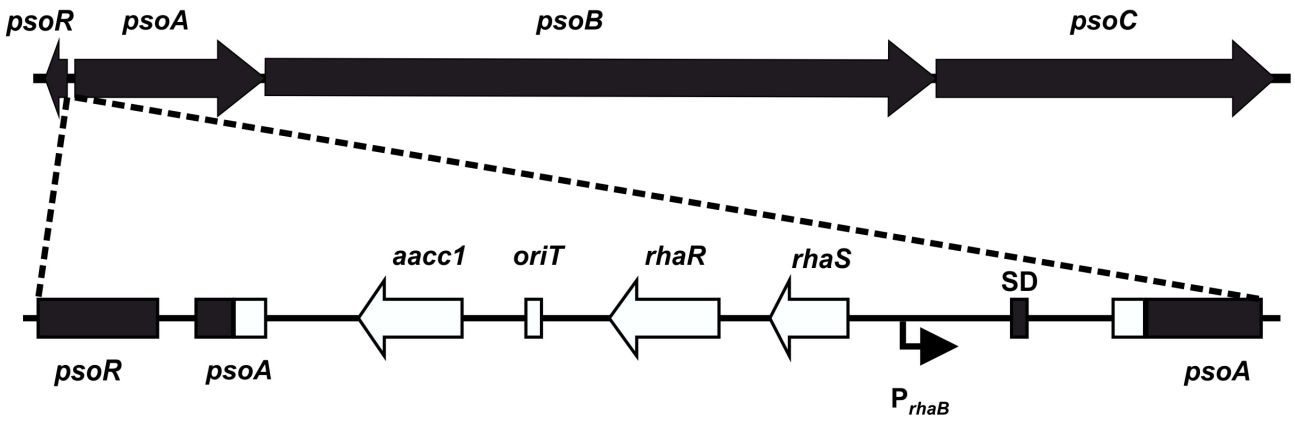
2.6 Supplementary Information

2.6.1 Supplementary Figures



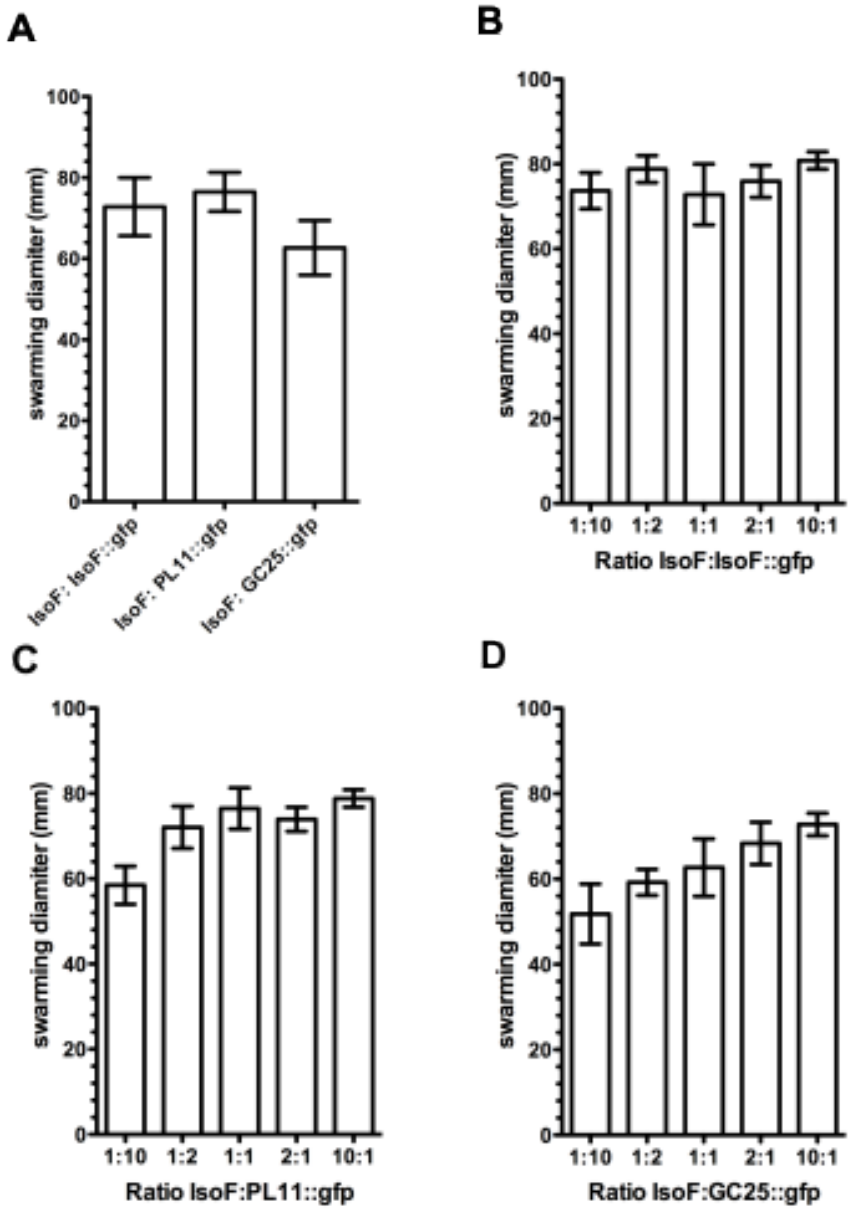
Supplementary Fig. 1.

Exposure to saturating concentration of AHL does not affect stochastic AHL-induction at early stages of biofilm development of *P. putida* IsoF. Flow cells were initiated with IsoF carrying the GFP-based AHL sensor**.** (A) After microcolonies were formed, only a few cells start to produce AHLs. Furthermore, the appearance of induced-single-cells in the proximity of microcolonies suggests they left the colony once activated. (B) When the experiment was performed under uniform amount of autoinducer in the medium, 0.5 μM 3-oxo-C10-HSL, a similar pattern in terms of induction heterogeneity was observed, although induction occurred earlier and in a higher proportion of cells.: 5 μm.

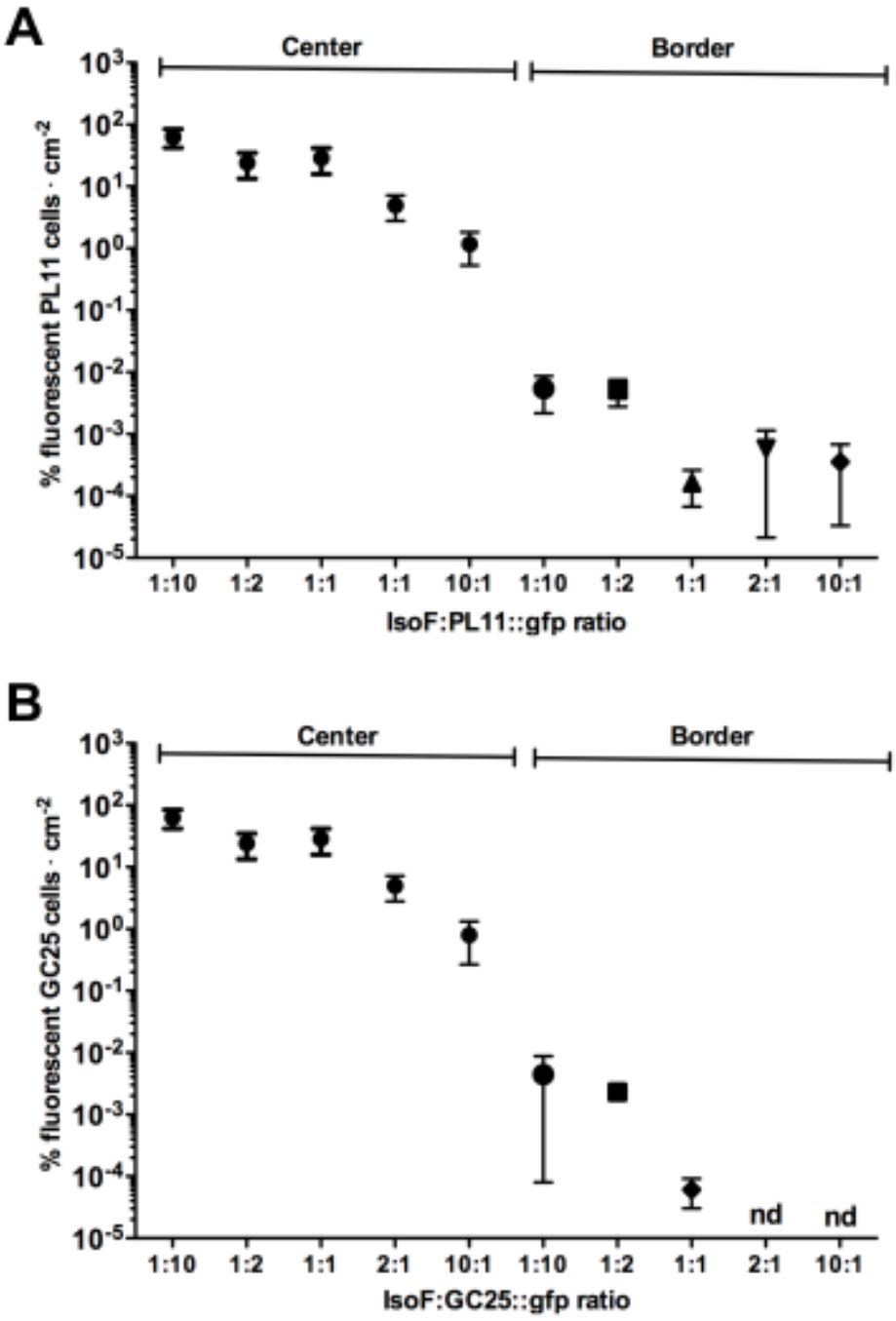


Supplementary Fig. 2.

Structure of putisolvin biosynthetic cluster and schematic outline of the construction of the conditional *psoABC* mutant PL2, in which natural *psoA* promoter has been replaced by the rhamnose-inducible promoter $P_{rhaB.}$ In addition, the strain carries genes required for the activation of the P_{rhaB} promoter (*rhaS* and *rhaR*); aacC1, aminoglycoside acetyltranferase (gentamycin resistance); *oriT*, origin of transfer; SD, Shirne-Delgarno.



Supplementary Fig. 3. Swarming motility of mixed cultures. The wild type (IsoF) was combined with itself, the putisolvin-deficient PL11, or the flagella-deficient mutant GC25 strain on swarming plates. *(A)* In 1:1 mixes, the displacement of the swarming colony remains unaffected irrespective of the strain mixture. When mixing IsoF at different ratios with: (*B*) itself, IsoF::gfp; (*C*) with PL11::gfp; or (*D*) with GC25::gfp, the swarming performance was unaffected across most mixing ratios. Only when the proportion of IsoF was decreased to 10%, swarming displacement is slightly reduced in mixtures with PL11::gfp and GC25::gfp. These findings support our conclusions that PL11::gfp and GC25::gfp cannot swarm along with IsoF wild type. Mean values of three independent experiments are shown with s.e.m.



Supplementary Fig. 4. Putisolvins are not public goods in swarming colonies. In mixed colonies with IsoF, mutants deficient for (*A*) putisolvin (PL11:gfp) and (*B*) flagella (GC25:gfp) production remain located in the center of the plate, while IsoF swarms to the edges. This pattern remained consistent across a wide range of mixing ratios, confirming that putisolvin is a private good under swarming conditions. Mean values of three independent experiments are shown with s.e.m.

2.6.2 Supplementary Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
E. coli		
MT102	*araD139* (*ara*-leu)7679 Δ*lac thi hsdR*	Laboratory collection
CC118(λpir)	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1* λ*pir* lysogen	(Herrero et al., 1990)
HB101	*recA thi pro leu hsd M*$^+$; Sm^R	(Boyer and Roulland-Dussoix, 1969)
P. putida		
IsoF	wild type; isolated from tomato roots; AHL$^+$	(Steidle et al., 2001)
F117	*ppuI* mutant of IsoF; Km^R	(Steidle et al., 2002)
GC3	*ppuR* mutant of IsoF; Km^R	This Laboratory
PL11	*psoA* mutant of IsoF; Gm^R	This study
GC25	*fliM* mutant of IsoF; Gm^R	This study
PL2	conditional *psoA* mutant of IsoF; P_{rhaB}- *psoA*; Gm^R	This study
Plasmids		
pEX18	gene replacement vector; Gm^R	(Hoang et al., 1998)
pEX18psoA	pEX18 containing an internal fragment of *psoA* in the SmaI site; Gm^R	This study
pSHAFT2	pUTmini-Tn*5*Cm with deleted BglII fragment; contains the *tnp* gene and I end of mini-Tn*5*; Gm^R	This laboratory
pSHAFT2fliM	pSHAFT2 containing an internal fragment of *fliM* in the StuI site; Gm^R	This study
pSC200	*ori*R6K; rhamnose-inducible promoter P_{RhaB}; Tp^R	(Ortega et al., 2007)
pSC200Gm	Gm^R resistance cassette cloned into XbaI/KpnI sites of pSC200; Gm^R	This study
pPO1	pSC200Gm carrying internal *psoA* fragment; Gm^R	This study
pPLlas	pUT/mini with Km^r::*lasR*-P_{lac}-P_{lasB}-*gfp*(ASV)-T_0-T_1 in the NotI site	(Lumjiaktase et al., 2010)
pRPL4las	RP4 with Km^R::*las*R-P_{lac}-P_{lasB}-*gfp*(ASV)	(Lumjiaktase et al., 2010)
pGA-G1	Promoter probe vector carrying a promoterless *gfp*-mut3 gene; Gm^R	(Schwager et al., 2012)
pLUM1	P_{psoA} promoter cloned into pGA-G1; Gm^R	This study
pLUM3	pBBR1MCS-3 carrying a P_{psoA}::*cfp* fusion; Tc^R	This study
pBAH8	$P_{A1/04/03}$-*gfp*mut3 fusion in pBBR1MCS-2; Km^R	(Huber et al., 2002)
pBBR1MCS-3	Broad-host-range plasmid, *lac*Zα; Tc^R	(Kovach et al., 1995)
pBBR1MCS-5	Broad-host-range plasmid, *lac*Zα; Gm^R	(Kovach et al., 1995)
pRK600	ColE1 RK2-Mob$^+$ RK2-Tra$^+$; helper plasmid; Cm^R	(de Lorenzo and Timmis, 1994)

2.7 References Results I

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3 Two quorum sensing-regulated loci are required for the biosynthesis of putisolvin biosurfactants in *Pseudomonas putida* IsoF

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Running title: Biosynthesis of putisolvin biosurfactants

3.1 Introduction

The production of *N*-acylhomoserine lactone (AHL) signal molecules by *Pseudomonas putida* is a strain-specific trait (Berg et al., 2002). The first AHL-dependent QS system was identified in *P. putida* IsoF, a strain that was isolated from the rhizosphere of tomato plants (Steidle et al., 2002; Steidle et al., 2001). This QS system is located on an genomic island that encodes four proteins: The AHL synthase PpuI, which directs the biosynthesis of 3-oxo-C10 and 3-oxo-C12 as major products; the AHL receptor PpuR; RsaL, which is encoded by a gene in the intergenic region between *ppuI* and *ppuR* and has been shown to repress expression of *ppuI* (Rampioni et al., 2012); and the AHL-regulated protein PpuA, which is of unknown function but shows similarity to long chain fatty acid CoA ligases from various organisms. It has been demonstrated that the IsoF wild type strain formed a homogenous biofilm, whereas a *ppuI* or a *ppuA* mutant formed structured biofilms with characteristic microcolonies and water-filled channels (Steidle et al., 2002).

Subsequent studies identified the *ppu* locus also in the plant growth promoting strains WCS358 (Bertani and Venturi, 2004), which was isolated from the rhizosphere of a potato plant (Geels and Schippers, 1983), and in the naphthalene degrader PCL1445 (Dubern et al., 2006), which was isolated from the roots of plants grown on a site polluted with polycyclic aromatic hydrocarbons (Kuiper et al., 2002). Inactivation of the ppu QS system in these strains strongly affected biofilm formation of PCL1445 but not of WCS358 (Bertani and Venturi, 2004; Dubern et al., 2006). A detailed analysis of strain PCL1445 revealed that the ppu QS system controls the biosynthesis of two powerful cyclic lipopeptide (CLPs) biosurfactants, putisolvin I and II. The two putisolvins have a similar structure and consist of a 12 aa peptide linked to a hexanoic lipid chain. The difference between the two biosurfactants is that putisolvin I has a valine at position eleven while putisolvin II has a leucin or isoleucine at this position. A large nonribosomal peptide synthethase, which is encoded by three genes, *psoA, psoB* and *psoC,* was demonstrated to be required for the biosynthesis of the 12 amino acids putisolvin peptide moiety (Fig. 1; (Dubern et al., 2008). Putisolvins were found to inhibit biofilm formation and were shown to even break down existing *Pseudomonas aeruginosa* biofilms (Kuiper et al., 2004). Dubern *et al.* (2006) also demonstrated that *ppu* QS mutants produce denser biofilms than the wild type strain because of decreased production of putisolvins. More recently, it has been shown that the pso locus is also present in *P. putida* IsoF and that in this strain the ppu QS system controls stochastic production of putisolvins in individual

cells in young biofilms (Cárcamo-Oyarce et al., 2015). In this study it was observed that the putisolvin biosurfactants remain associated with the envelope of the producing cell, rather than being shared as a public good, resulting in asocial motility of individual cells out of biofilm microcolonies.

In this study we show that *ppuA* encodes a fatty acid CoA ligase that is required to attach the hexanoic acid chain to the peptide moiety synthesized by the *psoABC* operon. Our results suggest that the peptide moiety alone has biological activity. However, its activity is boosted when the fatty acid is attached to the molecule. Furthermore, we show that expression of both operons is coordinated by the ppu QS system. Specifically, we demonstrate that PpuR directly controls expression of ppuA and indirectly of *psoABC via* expression of the transcriptional activator PsoR located upstream of *psoABC*.

3.2 Results

3.2.1 The *ppuI-rsaL-ppuR* QS system controls putisolvin biosynthesis indirectly through the LuxR-type regulator PsoR.

Previous work has demonstrated that expression of the *pso* gene cluster is regulated by the *ppuI-rsaL-ppuR* QS system (Cárcamo-Oyarce et al., 2015; Dubern et al., 2006). Furthermore, a gene encoding the LuxR-type regulator PsoR was identified upstream of *psoA* and was shown to be required for expression of the *pso* cluster (Dubern et al., 2008). Within the intergenic region Dubern et al. (2006) also identified a *lux* box, suggesting that PpuR is involved in the regulation of the expression of *psoA* or *psoR* or both. To address this issue we constructed a P_{psoR}-*gfp* transcriptional fusion and measured GFP fluorescence in the wild type IsoF, the *ppuI* mutant F117, the *ppuR* mutant GC3 and the *psoR* mutant GC4. These measurements showed that expression of *psoR* was strongly decreased in F117 and GC3 (Fig. 11A). However, expression could be restored to the wild-type level for F117, but not for GC3, when the medium was supplemented with 5 µM 3-oxo-C10-HSL. These results show that transcription of *psoR* is positively regulated by the *ppuI-rsaL-ppuR* QS system. To test the role of PsoR in the expression of *psoA* we transferred a P_{psoA}-*gfp* transcriptional fusion to mutant GC4. The expression of *psoA* was found to be reduced in the *psoR* mutant background relative to the wild type, suggesting that PsoR is a downstream regulator of the QS system (Fig. 11B).

In summary, our data suggest that production of putisolvins in strain IsoF is controlled by a regulatory cascade in which QS regulates expression of PsoR, which, in turn, activates transcription of the putisolvins biosynthetic genes (Fig. 12).

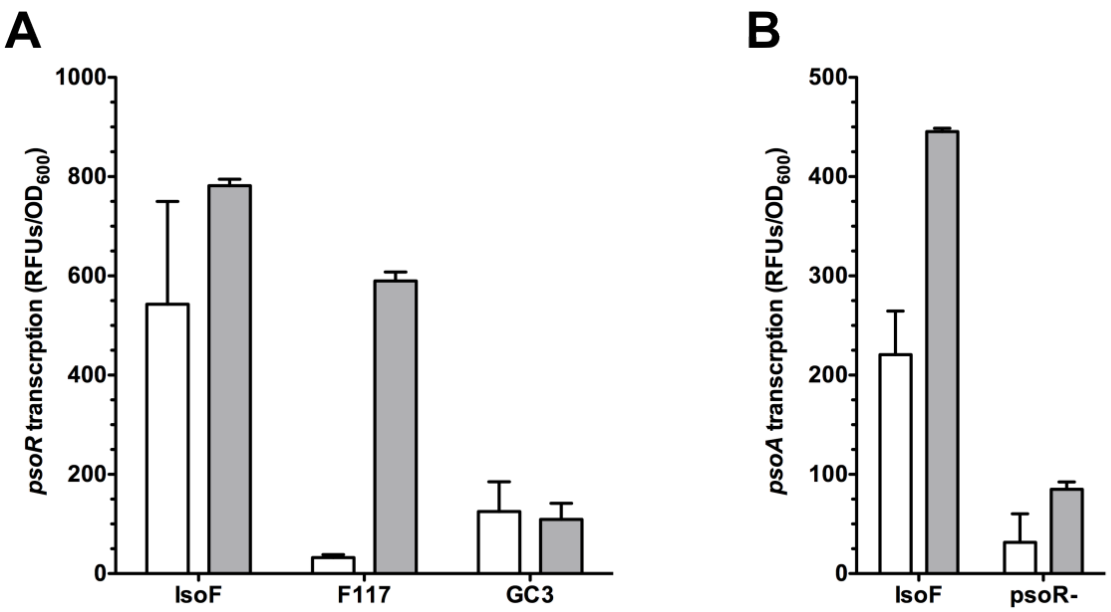

Figure 11. QS controls pso cluster expression through the LuxR-type regulator PsoR.

When measuring the *psoR* promoter activity (*A*) in ABC medium in the absence (open bars) or presence of 5 µM of 3-oxo-C10-AHL (gray bars), *psoR* promoter activity could be restored to wild-type IsoF level in the *ppuI* mutant F117 (which does not produce AHL signals), but not in the *ppuR* mutant GC3 (which cannot respond to AHL signals). When measuring the *psoA* promoter activity (*B*) *psoA* promoter activity could not be restored to wild-type IsoF level in the *psoR* mutant GC4. Mean values of three independent experiments are shown with s.d.

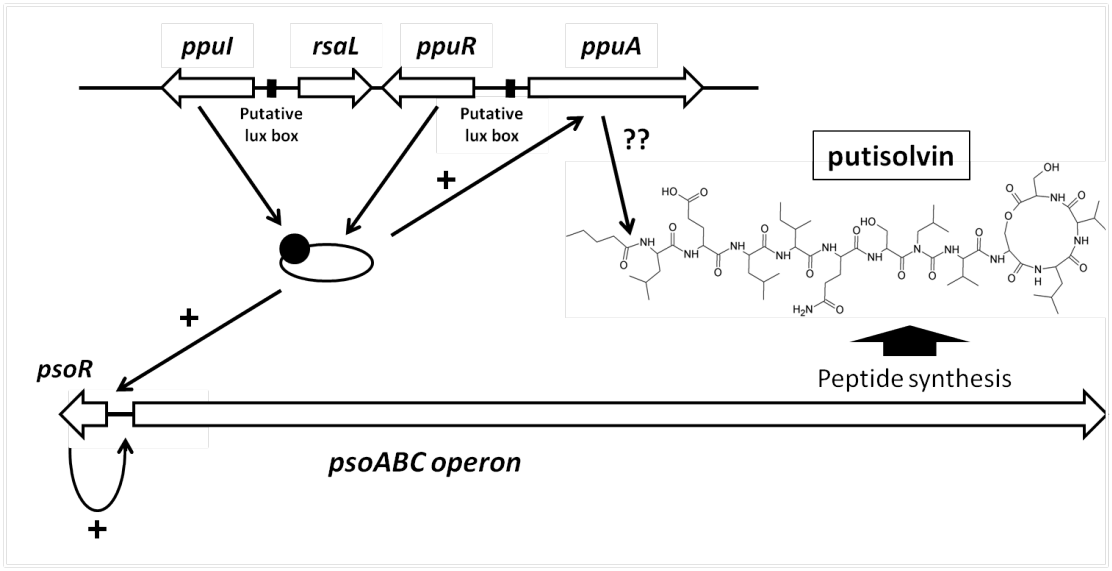

Fig 12. Model of QS regulation of putisolvin production in *P. putida* IsoF

3.3 PpuA is required for *N*-acylation of the putisolvin macrolactam core.

While Dubern *et al*. (2008) provided compelling evidence that the *psoACB* gene cluster directs the biosynthesis of the putisolvin peptide moiety it remained unclear how the molecule gets *N*-acylated at Leu1. Previous studies have shown that the assembly line for related *N*-acylated peptidolactams can be initiated in the presence of fatty acid coenzyme A (CoA) thioesters (Imker et al., 2010; Kraas et al., 2010; Steller et al., 2004). Sequence analysis revealed that no acyl CoA ligase is encoded within or in the vicinity of the putisolvin biosynthetic gene cluster ((Dubern et al., 2008); unpublished results). We therefore speculated that the putative long chain fatty acid CoA ligase PpuA could be involved in the biosynthesis of the activated fatty acid donor. PpuA is encoded by a gene that is not only located upstream of *ppuR* but is also stringently controlled by the PpuRI QS system (Steidle et al., 2002).

To assess the role of PpuA in putisolvin production we measured surfactant activities of culture supernatants of the wild type, the *ppuA* mutant D4 and the *psoA* mutant PL11 using the Du Nouy ring method (Fig. 13). In agreement with previous studies we observed that inactivation of the pso gene cluster strongly increases the surface tension of the culture supernatant (Cárcamo-Oyarce et al., 2015; Dubern et al., 2008). Likewise, inactivation of *ppuA* abolished surfactant activity, but the defect of mutant D4 could be rescued by introducing the wild-type allele on a plasmid.

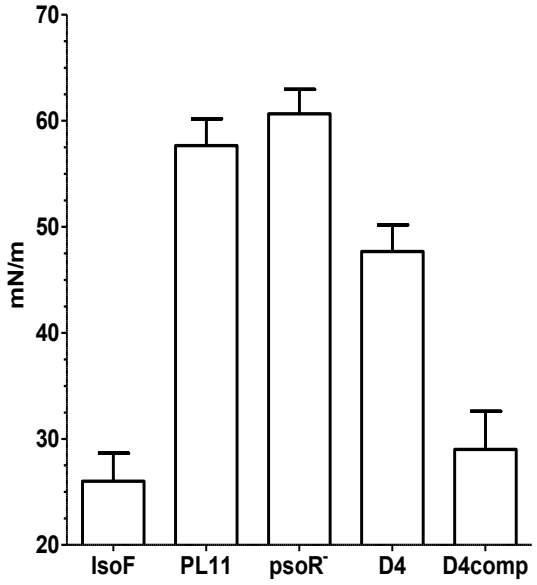

Fig 13. PpuA is involved in putisolvin biosynthesis.

When measuring the surface tensions from of spent cultures supernatants using the Du Nouy ring method, *ppuA* mutant D4, abolished surfactant activity, which is restored by introducing the wild-type allele on a plasmid. Mean values of three independent experiments are shown with s.d.

Extracts of culture supernatants of the different strains were also analyzed by high-performance liquid chromatography and mass spectrometry. While fractions of extracts of the wild type that showed surfactant activity contained two compounds with masses indicative of putisolvin I and II these molecules were not detectable in extracts of the *psoA* mutant. In conclusion, our data suggest that PpuA is required for the biosynthesis of CoA-activated hexanoic acid that serves as a donor for *N*-acylation of the putisolvin macrolactam.

3.4 PpuA is required for swarming motility.

We have recently shown that putisolvin production is essential for swarming motility of *P. putida* IsoF (Cárcamo-Oyarce et al., 2015). When tested for swarming motility by spotting cells onto the center of ABC plates solidified with 0,4 % agar the wild type IsoF colonized the entire plate within 3 days whereas the *ppuA* mutant D4 showed an approximately 50% reduction in swarm colony diameter relative (Fig 14). This defect could be rescued by genetic complementation. Under these conditions the *psoA* mutant PL11 showed no surface migration (Cárcamo-Oyarce et al., 2015), suggesting that while the peptide moiety of putisolvin is essential for swarming motility its activity is increased when the molecule is *N*-acylated.

A

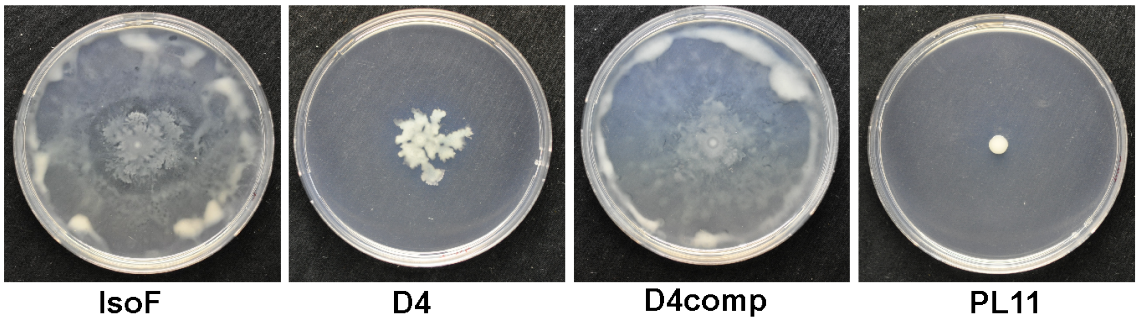

Figure 14. PpuA is required for swarming motility in IsoF.

Swarming motility was observed (A) and quantified (B) in ABC soft medium. Swarming of the *ppuA* mutant D4 was reduced, but restored to the wild type levels after genetic complementation of ppuA gene. PL11 is impaired in swarming because they do not produce putisolvin Results are representative of three independent experiments. Swarming plates were photographed after 2 days of incubation. Mean values of three independent experiments are shown with s.d.

3.5 Putisolvins exhibit antifungal activity.

Several CLPs have been demonstrated to not only act as surfactants but also exhibit antimicrobial activities (Raaijmakers et al., 2010). A previous study it reported that purified putisolvin biosurfactants from supernatants of *P. putida* 267 lyse zoospores of *Phytophthora capsici* and inhibit growth of the fungal pathogens *Botrytis cinerea* and *Rhizoctonia solani* (Kruijt et al., 2009). For this reason we tested antimicrobial activites of *P. putida* IsoF against a panel of bacteria and fungi. We observed that the wild type strain exhibited moderate activity against the fungus *Alternaria alternata* whereas the conditional *psoA* mutant PL2, in which the native promoter region of *psoA* has been replaced with the rhamnose-inducible P_{rhaB} promoter, did not. However, antifungal activity could be restored to the level of the wild type when rhamnose was added to the medium (Fig. 15A). We next investigated the role of *ppuA* in antifungal actvity of IsoF. In contrast to the psoA mutant, the *ppuA* mutant D4 exhibited approximately 50% of the antifungal activity of the wild type strain. Complementation with the wild type allele *in trans* fully restored the activity to the level of the wild type (Fig. 15D). These results suggest, that the putisolvin peptide moiety is antifungal but that its activity is boosted when the molecule is *N*-acylated.

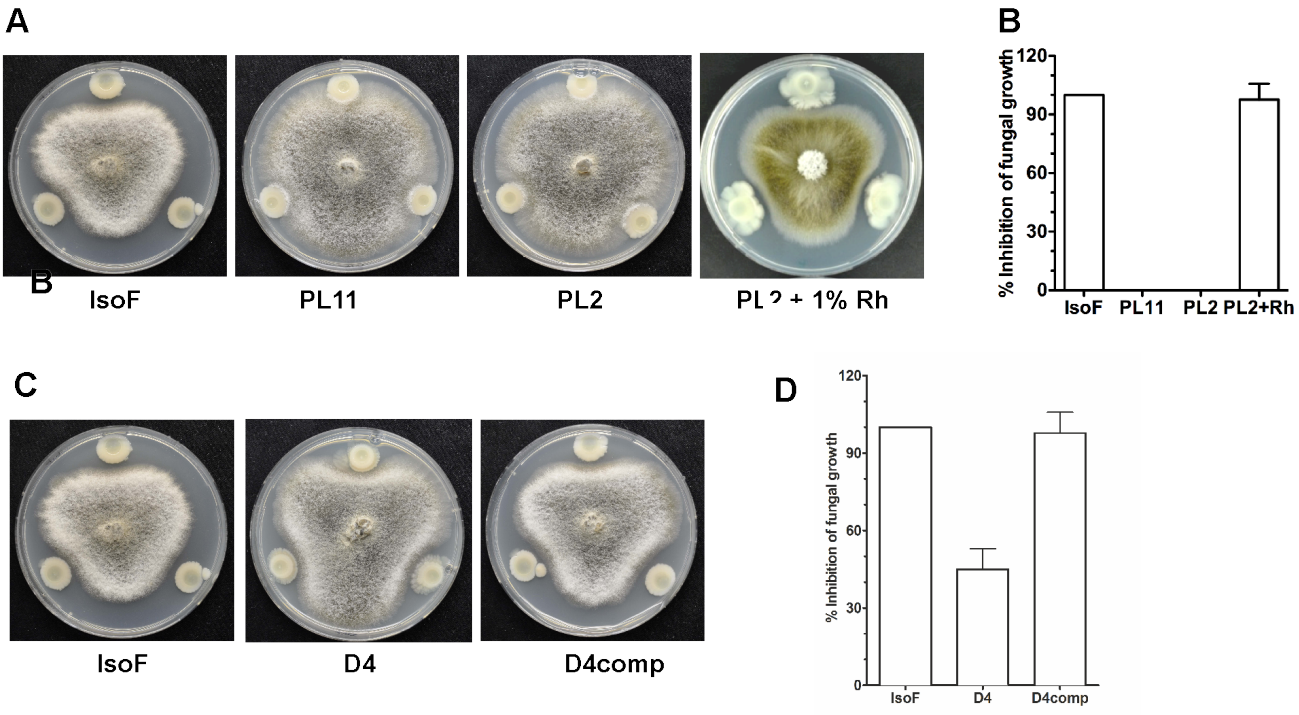

Figure 15. Putisolvin mediates antifungal activity in *P. putida*.

(A) Antifungal activity of the conditional *psoA* mutant PL2 was abolished on MEA plates, but was increasingly restored on plates supplemented with 0.5%, 1% or 2% rhamnose. (B) Antifungal activity of the *ppuA* mutant D4 was reduced, but restored to the wild type levels after genetic complementation of ppuA gene Pictures were taken after 5-6 days of incubation. Mean values of three independent experiments are shown with s.d.

3.6 Conclusions

In this study we show that PpuA directs the biosynthesis of CoA-activated hexanoic acid which serves as a donor for chain-initiating N-acylation in putisolvin biosynthesis. This mechanism of lipoinitiation has previously reported for the biosynthesis of surfactin and glidobactin and has been suggested to be a wide-spread mechanism for the N-acylation of lipopeptides (Imker et al., 2010; Kraas et al., 2010). In the case of surfactin biosynthesis it has been shown that host-encoded fatty acyl CoA ligases (FACL) synthesize the 3-hydroxy fatty acyl-CoA that is required for surfactin biosynthesis (Kraas et al., 2010). Likewise it has been assumed that the glidobactin assembly line uses host-derived fatty acid CoA donors (Imker et al., 2010). In this study we demonstrate that the biosynthesis of the lipopeptide putisolvin depends on the activity of the dedicated FACL PpuA. The *ppuA* gene is not part of the *P. putida* core genome but resides on a genomic island that also contains the *ppuI-rsaL-ppuR* QS system and is located in the intergenic region between *suhB* and *PA3819* (Steidle et al., 2002). A BLAST search revealed that this island is only present in a few *Pseudomonas* strains, namely *P. putida* IsoF, PCL1445 and WCS358 and *Pseudomonas* sp. H2 and LAIL14HWK12:I7. The proteins encoded on these islands share at least 99% sequence identity. All strains containing the ppu locus also contain the putisolvin biosynthesis cluster and at least for strains PCL1445 and IsoF it has been demonstrated that expression of *ppuA* and the *pso* gene cluster is co-regulated by the *ppuI-rsaL-ppuR* QS system; Fig. 1). There is one interesting exception to this rule: *P. putida* strain W15Oct28 contains the pso gene cluster but misses the ppu island; yet, by using high-resolution and CID spectrometry it has been demonstrated that this strain produces compounds with molecular weights and aa sequences identical to the two putisolvins. As no *ppuA* homolog could be identified in the genome sequence of this organism, it must be assumed that in this strain a yet unidentified FACL replaces the activity of PpuA. It is also interesting to note that the lux box that is present in the psoA-psoR intergenic region that is present in all strains harboring the ppu island is missing in strain W15Oct28 (data not shown).

A phylogenetic analysis of NRPS domains of systems directing the biosynthesis of structurally very different lipopeptides (as well as their linked regulators and tripartite export systems) in *Pseudomonas* sp. revealed an unexpected high degree of evolutionary relatedness (Li et al., 2013; Fig. 16). The authors of this study suggested that pseudomonads may have recruited and

reshuffled individual biosynthetic modules to engineer novel NRPS assembly lines for the synthesis of structural lipopeptide variants with different biological functions. While the peptide moieties of these molecules are different they are all *N*-acylated at the first amino acid, generally with a hydroxy fatty acid and very often with 3-hydroxydecanoic acid. It has been proposed that these hydroxy fatty acids are produced by the primary metabolism, likely by the type II fatty acid synthase systems (Gross et al., 2007; Kraas et al., 2010). Given that putisolvin is the only *Pseudomonas* lipopeptide which carries a hexanoic acid at the N-terminus it is tempting to speculate that in most *Pseudomonas* strains the amount of CoA-activated hexanoic acid is too low for putisolvin production. For such a strain the acquisition of the ppu island is essential to efficiently produce putisolvin. This functional interdependency may also provide an explanation for the co-regulation of the two loci.

At present it is unclear how specific the interaction of PpuA with the putisolvin starter C domain is. It will be of great interest to explore whether the enzyme can possibly also cooperate with starter C domains of other NRPS complexes. This possibility could open novel avenues for the design of lipopeptides with altered fatty acid chain length and thus changed biological activities.

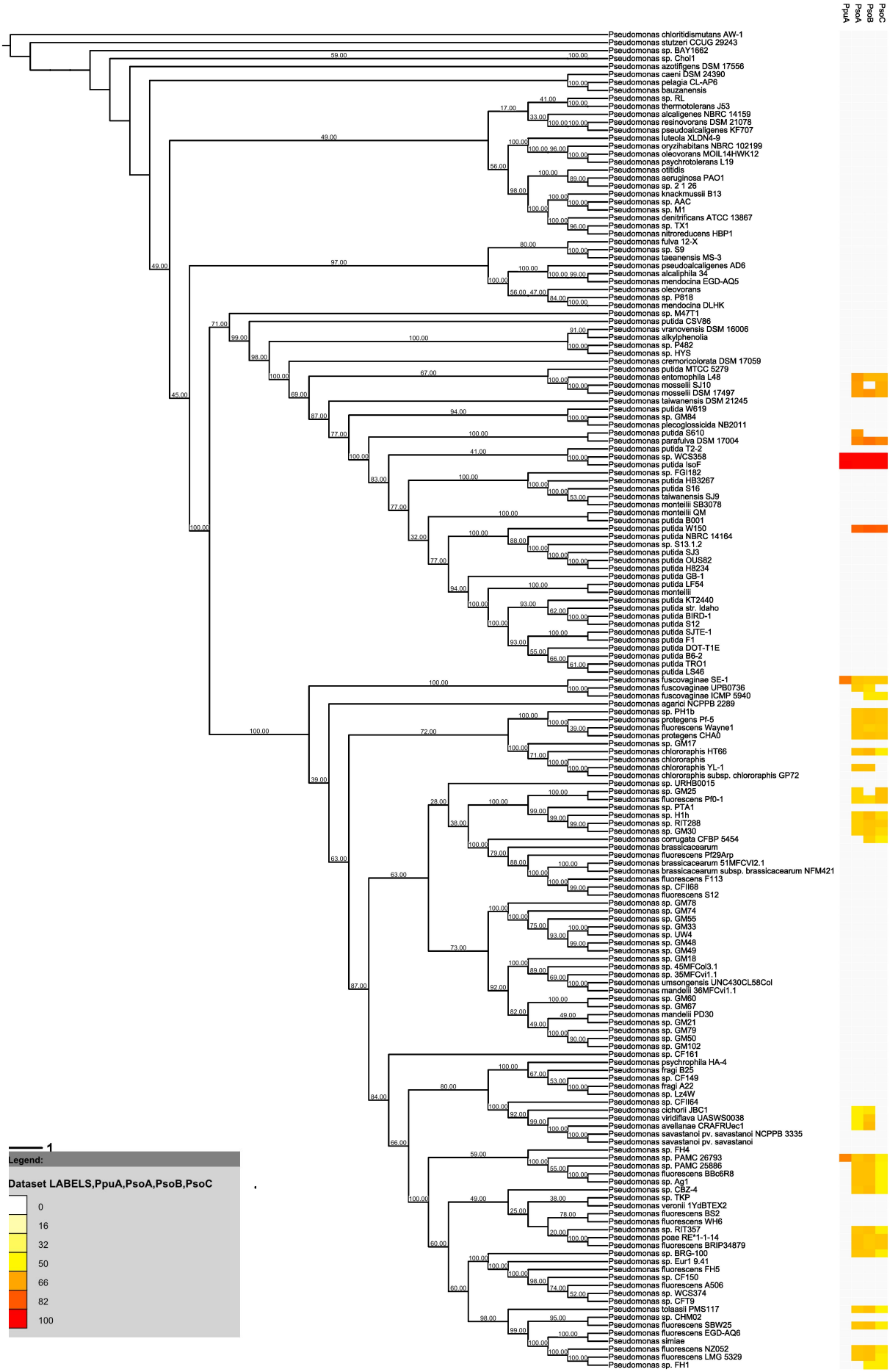
PpuA
PsoA
PsoB
PsoC
Pseudomonas chloritidismutans AW-1
Pseudomonas stutzeri CCUG 29243
Pseudomonas sp. BAY1662
Pseudomonas sp. Chol1
Pseudomonas azotifigens DSM 17556
Pseudomonas caeni DSM 24390
Pseudomonas pelagia CL-AP6
Pseudomonas bauzanensis
Pseudomonas sp. RL
Pseudomonas thermotolerans J53
Pseudomonas alcaligenes NBRC 14159
Pseudomonas resinovorans DSM 21078
Pseudomonas pseudoalcaligenes KF707
Pseudomonas luteola XLDN4-9
Pseudomonas oryzihabitans NBRC 102199
Pseudomonas oleovorans MOIL14HWK12
Pseudomonas psychrotolerans L19
Pseudomonas otitidis
Pseudomonas aeruginosa PAO1
Pseudomonas sp. 2 1 26
Pseudomonas knackmussii B13
Pseudomonas sp. AAC
Pseudomonas sp. M1
Pseudomonas denitrificans ATCC 13867
Pseudomonas sp. TX1
Pseudomonas nitroreducens HBP1
Pseudomonas fulva 12-X
Pseudomonas sp. S9
Pseudomonas taeanensis MS-3
Pseudomonas pseudoalcaligenes AD6
Pseudomonas alcaliphila 34
Pseudomonas mendocina EGD-AQ5
Pseudomonas oleovorans
Pseudomonas sp. P818
Pseudomonas mendocina DLHK
Pseudomonas sp. M47T1
Pseudomonas putida CSV86
Pseudomonas vranovensis DSM 16006
Pseudomonas alkylphenolia
Pseudomonas sp. P482
Pseudomonas sp. HYS
Pseudomonas cremoricolorata DSM 17059
Pseudomonas putida MTCC 5279
Pseudomonas entomophila L48
Pseudomonas mosselii SJ10
Pseudomonas mosselii DSM 17497
Pseudomonas taiwanensis DSM 21245
Pseudomonas putida W619
Pseudomonas sp. GM84
Pseudomonas pleoglossicida NB2011
Pseudomonas putida S610
Pseudomonas parafulva DSM 17004
Pseudomonas putida T2-2
Pseudomonas sp. WCS358
Pseudomonas putida IsoF
Pseudomonas sp. FGI182
Pseudomonas putida HB3267
Pseudomonas putida S16
Pseudomonas taiwanensis SJ9
Pseudomonas monteilii SB3078
Pseudomonas monteilii QM
Pseudomonas putida B001
Pseudomonas putida W150
Pseudomonas putida NBRC 14164
Pseudomonas sp. S13.1.2
Pseudomonas putida SJ3
Pseudomonas putida OUS82
Pseudomonas putida H8234
Pseudomonas putida GB-1
Pseudomonas putida LF54
Pseudomonas monteilii
Pseudomonas putida KT2440
Pseudomonas putida str. Idaho
Pseudomonas putida BIRD-1
Pseudomonas putida S12
Pseudomonas putida SJTE-1
Pseudomonas putida F1
Pseudomonas putida DOT-T1E
Pseudomonas putida B6-2
Pseudomonas putida TRO1
Pseudomonas putida LS46
Pseudomonas fuscovaginae SE-1
Pseudomonas fuscovaginae UPB0736
Pseudomonas fuscovaginae ICMP 5940
Pseudomonas agarici NCPPB 2289
Pseudomonas sp. PH1b
Pseudomonas protegens Pf-5
Pseudomonas fluorescens Wayne1
Pseudomonas protegens CHA0
Pseudomonas sp. GM17
Pseudomonas chlororaphis HT66
Pseudomonas chlororaphis
Pseudomonas chlororaphis YL-1
Pseudomonas chlororaphis subsp. chlororaphis GP72
Pseudomonas sp. URHB0015
Pseudomonas sp. GM25
Pseudomonas fluorescens Pf0-1
Pseudomonas sp. PTA1
Pseudomonas sp. H1h
Pseudomonas sp. RIT288
Pseudomonas sp. GM30
Pseudomonas corrugata CFBP 5454
Pseudomonas brassicacearum
Pseudomonas fluorescens Pf29Arp
Pseudomonas brassicacearum 51MFCVI2.1
Pseudomonas brassicacearum subsp. brassicacearum NFM421
Pseudomonas fluorescens F113
Pseudomonas sp. CFII68
Pseudomonas fluorescens S12
Pseudomonas sp. GM78
Pseudomonas sp. GM74
Pseudomonas sp. GM55
Pseudomonas sp. GM33
Pseudomonas sp. UW4
Pseudomonas sp. GM48
Pseudomonas sp. GM49
Pseudomonas sp. GM18
Pseudomonas sp. 45MFCol3.1
Pseudomonas sp. 35MFCvi1.1
Pseudomonas umsongensis UNC430CL58Col
Pseudomonas mandelii 36MFCvi1.1
Pseudomonas sp. GM60
Pseudomonas sp. GM67
Pseudomonas mandelii PD30
Pseudomonas sp. GM21
Pseudomonas sp. GM79
Pseudomonas sp. GM50
Pseudomonas sp. GM102
Pseudomonas sp. CF161
Pseudomonas psychrophila HA-4
Pseudomonas fragi B25
Pseudomonas sp. CF149
Pseudomonas fragi A22
Pseudomonas sp. Lz4W
Pseudomonas sp. CFII64
Pseudomonas cichorii JBC1
Pseudomonas viridiflava UASWS0038
Pseudomonas avellanae CRAFRUec1
Pseudomonas savastanoi pv. savastanoi NCPPB 3335
Pseudomonas savastanoi pv. savastanoi
Pseudomonas sp. FH4
Pseudomonas sp. PAMC 26793
Pseudomonas sp. PAMC 25886
Pseudomonas fluorescens BBc6R8
Pseudomonas sp. Ag1
Pseudomonas sp. CBZ-4
Pseudomonas sp. TKP
Pseudomonas veronii 1YdBTEX2
Pseudomonas fluorescens BS2
Pseudomonas fluorescens WH6
Pseudomonas sp. RIT357
Pseudomonas poae RE*1-1-14
Pseudomonas fluorescens BRIP34879
Pseudomonas sp. BRG-100
Pseudomonas sp. Eur1 9.41
Pseudomonas fluorescens FH5
Pseudomonas sp. CF150
Pseudomonas fluorescens A506
Pseudomonas sp. WCS374
Pseudomonas sp. CFT9
Pseudomonas tolaasii PMS117
Pseudomonas sp. CHM02
Pseudomonas fluorescens SBW25
Pseudomonas fluorescens EGD-AQ6
Pseudomonas simiae
Pseudomonas fluorescens NZ052
Pseudomonas fluorescens LMG 5329
Pseudomonas sp. FH1
1
Legend:
Dataset LABELS,PpuA,PsoA,PsoB,PsoC
0
16
32
50
66
82
100

Figure 16. Distribution of the *pso* cluster and *ppuA* gene in *Pseudomonas*.

Maxium likelihood phylogeny was reconstructed as described in Materials and Methods. Support values (100 bootstraps) are given at the nodes. Whole genome sequences were searched for the *ppuA*, and *psoABC* genes using the respective protein sequences from *Pseudomonas putida* IsoF as described in Materials and Methods. Heatmap colors depict the tblastn identity scores, from red 100% identity to white 0% identity.

3.7 Materials and Methods

3.7.1 Strains, Media and Culture Conditions

Bacterial strains and plasmids used in this study are listed in Table 2**.** All strains were grown aerobically in Luria–Bertani (LB) at 30°C (*Pseudomonas putida*) or 37°C (*Escherichia coli*). *P. putida* transconjugants were isolated on Pseudomonas Isolation Agar (PIA) at 30°C. In most experiments *P. putida* strains were grown in modified AB medium supplemented with 10 mM sodium citrate (Heydorn et al., 2000) (referred henceforward as ABC medium). Antibiotics were added as required at final concentrations of: for *E. coli*: 100 µg ml^{-1} ampicilin, 50 µg ml^{-1} kanamycin, 10 µg ml^{-1} gentamycin; for *P. putida*: 100 µg ml^{-1} kanamycin and 20 or 50 µg ml^{-1} gentamycin

3.7.2 Construction of *P. putida* IsoF mutants

The *psoR* mutant GC3 was generated as follows: an internal *psoR* fragment was PCR amplified using the primers psoRF (5'-ctgatggtgtcgttcgaagagg-3') and psoRR (5'-gctcgtcgagcacgtacaactg-3') and cloning the PCR product blunt-end into the StuI-digested gene replacement vector pSHAFT2Gm. The resulting plasmid, pSHAFT2*psoR,* was mobilized into *P. putida* IsoF by triparental mating and gene replacement mutants were selected on PIA medium containing 20 µg ml^{-1} gentamycin. The genetic structures of all mutants constructed were confirmed by PCR and sequence analysis.

3.7.3 Construction of transcriptional fusions

To construct a P_{psoR}-*gfp* transcriptional fusion the *psoR* promoter region was PCR amplified using the primers p-psoRF (5'-aggatccgattctaagctttgcggcg-3') and p-psoRR (5'-tggatccgctcagggcaaaggtttcg-3'). PCR fragments were cloned as *BamHI* fragments (restriction sites are underlined) into the respective site of the promoter-probe vector pGA-G1, generating the plasmids pPLM2 (P_{psoR}-*gfp).* The plasmids were mobilized from *E. coli* CC118 to *P. putida* strains by conjugation and selected on PIA medium supplemented with 50 µg ml^{-1} gentamycin (pPLM1) or 100 µg ml^{-1} tetracycline (pLUM3).

3.7.4 Measurement of promoter activities

P. putida strains harboring pPLM2 were grown in 10 mM ABC medium for 24 hrs at 30°C with continuous shaking. When required 3-oxo-C10 homoserine lactone was added to the medium at a final concentration of 5 µM. Green fluorescence was measured using 200 µl samples in a microtitre plate reader (SynergyTM HT, MWG Biotech, Germany) with an excitation wavelength of 485 nm and emission detection at 528 nm. The data were corrected for autofluorescence and processed with the KC4 software (BioTek Instruments). Specific fluorescence was calculated by normalizing relative fluorescence to OD_{600}, which was simultaneously measured in the instrument.

3.7.5 Swarming Motility assays

Swarming motility was determined on ABC agar plates supplemented with 0.1% casamino acids and solidified with 0.4% (wt/vol) agar as described previously (Eberl et al., 1996). Briefly, overnight cultures were adjusted to an OD_{600} of 0.1 and 2 µl samples were inoculated on the center of swarming plates, which were incubated for 3 days at 30°C.

3.7.6 Antifungal activity

Antagonistic activities of bacterial strains against *Alternaria alternata* were preformed as previously described (Agnoli et al., 2012). Briefly, 20 µl stationary-phase cultures (adjusted OD600=1) of bacterial strains were spotted at three positions on on Malt agar (DIFCO) plates with 2% agar. Following incubation for one night at 30°C a 5-mm-diameter fungal inoculum, which was cut from the outer (youngest) edge of the fungal mycelium, was placed onto the center of the plate. Plates were incubated at RT in the dark and inhibition zones were recorded after 4 days.

3.7.7 Biosurfactant production

To quantify biosurfactant production the decrease of surface tension between culture medium and air was determined with a Du Nouy ring (Dubern et al., 2006).

3.7.8 Putisolvin extraction, HPLC

300 ml of bacterial culture were grown in ABC medium supplemented with glycerol (Dubern and Bloemberg, 2006). Putisolvin extraction and HPLC-MS chromatography were preformed as described previously (Kuiper et al., 2004)

3.7.9 In silico sequences analysis

Whole genome sequences of Pseudomonas species were downloaded from NCBI Genbank. The *rpoD*, *recA* and *gyrB* gene sequences were obtained for each genome and aligned using MAFFT v7.0 (Katoh and Standley, 2013). The nucleotide alignment was edited in the CLC Genomics Workbench (Qiagen). Maximum likelihood reconstruction was done using RAxML v8.0 (Stamatakis, 2014) with GAMMA nucleotide substitution model and 100 rapid bootstrap analyses. The PsoA, PsoB, PsoC and PpuA protein sequences of *Pseudomonas putida* IsoF were used to query the genomes using the NCBI tblastn software. Only hits with e-value < $1x10^{-15}$ and identity > 50% were considered. The phylogenetic data and the tblastn homology scores were loaded into iTOL (Letunic and Bork, 2011) for visualization.

Table 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
Strains		
E. coli		
MT102	*araD139* (*ara*-leu)7679 Δ*lac thi hsdR*	Laboratory collection
CC118(λpir)	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1* λ*pir* lysogen	(Herrero et al., 1990)
HB101	*recA thi pro leu hsd M*$^+$; Sm^R	(Boyer and Roulland-Dussoix, 1969)
P. putida		
IsoF	wild type; isolated from tomato roots; AHL$^+$	(Steidle et al., 2001)
F117	*ppuI* mutant of IsoF; Km^R	(Steidle et al., 2002)
D4	*ppuA* mutant of IsoF	(Steidle et al., 2002)
GC3	*ppuR* mutant of IsoF; Km^R	(Cárcamo-Oyarce et al., 2015)
PL11	*psoA* mutant of IsoF; Gm^R	(Cárcamo-Oyarce et al., 2015)
PL2	conditional *psoA* mutant of IsoF; P_{rhaB}-*psoA*; Gm^R	(Cárcamo-Oyarce et al., 2015)
GC4	*psoR* mutant of IsoF; Km^R	This study
Plasmids		
pSHAFT2	pUTmini-Tn*5*Cm with deleted BglII fragment; contains the *tnp* gene and I end of mini-Tn*5*; Gm^R	This laboratory
pGA-G1	Promoter probe vector carrying a promoterless *gfp*-mut3 gene; Gm^R	(Schwager et al., 2012)
pLUM1	P_{psoA} promoter cloned into pGA-G1; Gm^R	(Cárcamo-Oyarce et al., 2015)
pLUM2	P_{psoR} promoter cloned into pGA-G1; Gm^R	This study
pBBR1MCS-5	Broad-host-range plasmid, *lac*Zα; Gm^R	(Kovach et al., 1995)
pGC1	*ppuA* gene complementation colned into pBBR1MCS-5	This study
pRK600	ColE1 RK2-Mob$^+$ RK2-Tra$^+$; helper plasmid; Cm^R	(de Lorenzo and Timmis, 1994)

3.8 References Results II

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4 *Pseudomonas putida* IsoF is capable of invading biofilms by the aid of a type VI secretion system

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4.1 Introduction

It is well accepted that in most natural settings bacteria exist in multispecies biofilms (Costerton et al., 1999; Danhorn and Fuqua, 2007; Hall-Stoodley et al., 2004). Due to diffusion limitation nutrient gradients are present within biofilms, which create spatial niches for the different members of the biofilm consortium (Boles et al., 2004). However, within these niches the bacteria face a constant battle for limited space and resources (Hibbing et al., 2010). Because of the close association of bacteria within biofilms they have developed strategies to take advantage of beneficial interactions (Elias and Banin, 2012) and to prevent detrimental associations (Rendueles and Ghigo, 2012).

Bacteria have developed contact-dependent inhibition systems, which depend on effector proteins that impair growth of the targeted cell (Hayes et al., 2010). Among these contact-dependent systems, two are specially relevant for bacterial competition: i) the contact-dependent growth inhibition system (CDI), which is a type V secretion system that uses a long β-helical cell surface protein to bind to receptors in the target cells to deliver a growth inhibitory signal (Ruhe et al., 2013) and ii) the type VI secretion system (T6SS), which utilizes a bacteriophage-like apparatus to puncture the cell envelope to translocate protein effectors into both eukaryotic and prokaryotic target cells (Silverman et al., 2012). T6SS has been reported to mediate interbacterial antagonism in several species, and it is proposed to play an important role in the dynamics of bacterial communities (Russell et al., 2014). One of the best characterized T6SS belongs to the human pathogen *Pseudomonas aeruginosa* (Hood et al., 2010; Mougous et al., 2006), which mediates antibacterial activity by delivering effector proteins that degrade the peptidoglycan of target cells (Russell et al., 2011). By means of genomic analyzes, T6SS gene clusters have been identified in several *Pseudomonas* species, including *P. putida* (Barret et al., 2011; Boyer et al., 2009). In the case of *P. putida*, the biological role of the T6SS has not been analyzed. Here, we identified and characterized a T6SS in the plant growth-promoting bacterium *P. putida* IsoF. It is shown that this system antagonizes several proteobacterial species, including pseudomonads that inhabit similar niches as strain IsoF. Moreover, we show that IsoF antagonism is particularly effective in mixed-species biofilms. Our results extend the understanding of bacterial interactions in multispecies biofilms and provide evidence of the involvement of T6SS in biofilm defense and invasion.

4.2 Results

4.2.1 IsoF displays contact-dependent antagonism against Gram-negative bacteria

Contact-mediated interactions are particularly relevant in densely populated communities such as biofilms (Elias and Banin, 2012; Fuqua, 2010; Hibbing et al., 2010; Rendueles and Ghigo, 2012). *P. putida* IsoF was isolated from tomato roots and was shown to effectively persist in the rhizosphere of different plants grown in natural soil. To test how the strain interacts with possible members of the co-residing community we used a bacterial interaction model. To this end, *P. putida* IsoF was mixed in different ratios with the strains to test on an agar surface, such that an artificial mixed colony developed. To monitor the fate of the two strains they were engineered to express either the green fluorescent protein (GFP) or the red fluorescent protein (dsRed).

After incubating the mixed colonies for 24 h on ABC medium, IsoF was found to inhibit the growth (visualized by a decrease in green fluorescence) of members of the genera *Burkholderia* (*B. cenocepacia* H111, *B. cepacia*, *B. multivorans*) and *Pseudomonas* (*P. putida* KT2240, *P. fluorescens*, *P. aeruginosa*) as well as *Serratia liquefaciens* and *Escherichia coli*. By contrast, the viability of IsoF was not affected in any of these cases (Fig 17).

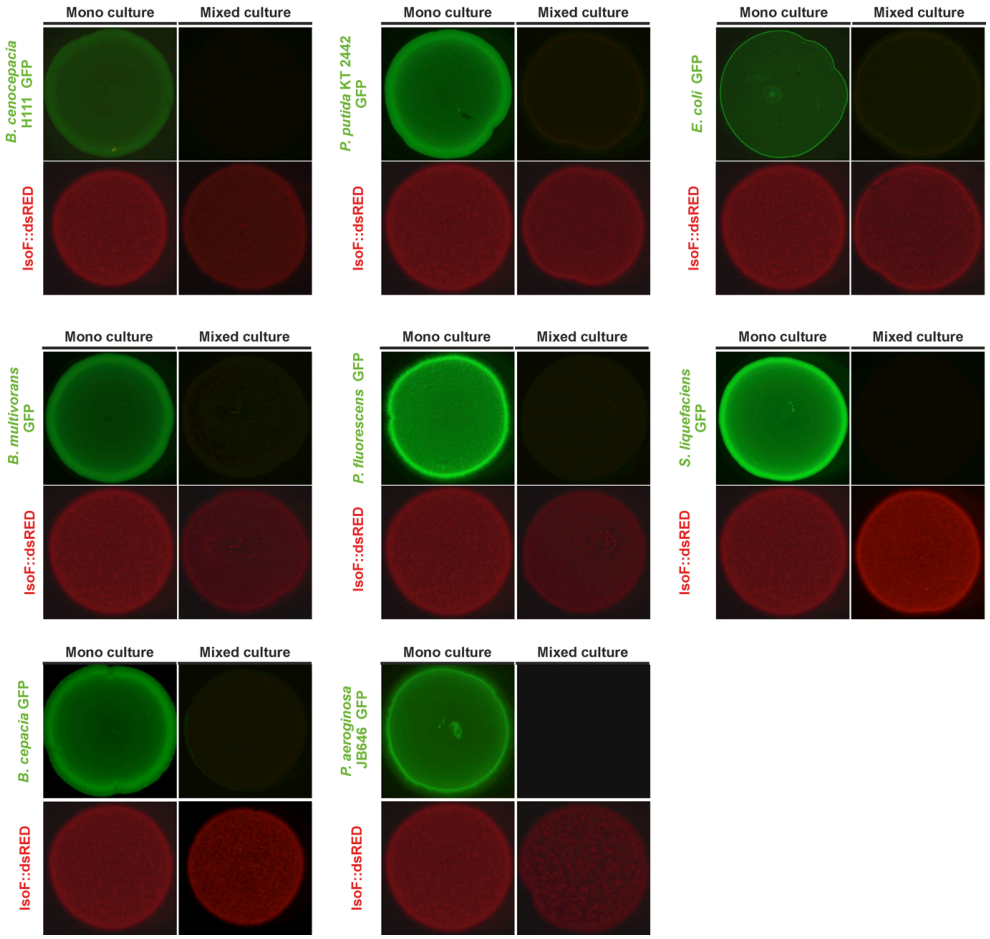

Figure 17. IsoF displays contact-dependent antagonism against Gram-negative bacteria.

Growth competition assays between the indicated GFP-labeled strains and dsRED-labeled IsoF.

4.2.2 Contact dependent antagonism is dose-dependent

To exclude that differences in bacterial growth between the tested strains and IsoF could lead to a bias in the competition outcomes, we selected strains that displayed similar growth kinetics to IsoF under the experimental settings used. *P. putida* KT2240, *P. chlororaphis, P. aureofaciens*, *P. fluorescens*, *P. entomophila* were selected for this purpose (Fig 18 A). Since bacterial growth can be affected by compounds released by other bacteria present in the environment (Reder-Christ et

al., 2012; Rendueles and Ghigo, 2012; Ron and Rosenberg, 2001), we tested the effect of spent culture supernatants of IsoF on growth of the selected strains. These assays showed that no antibacterial compounds were secreted by the strains (Fig 18 B). However, when grown as mixed colonies on agar, IsoF effectively inhibited the growth of the five selected *Pseudomonas* strains, in most cases causing a 3-5 log decrease in CFUs.

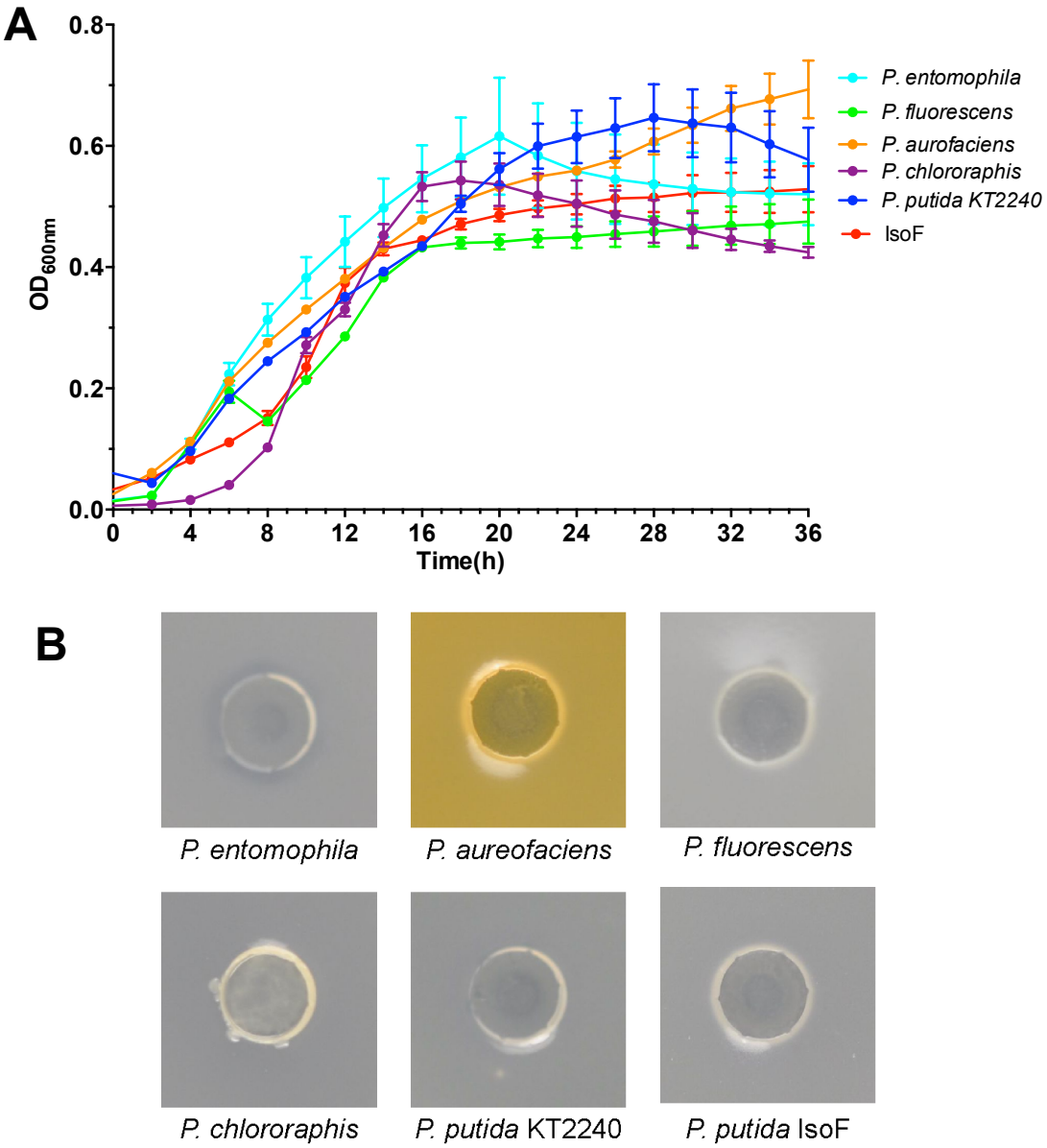

Figure 18.

(A) Growth of indicated *Pseudomonas* strains in liquid medium. (B) Growth of pseudomonads used in competition assays is not affected by diffusible compounds produced by IsoF.

Interestingly, when lowering the relative amount of IsoF cells in the inoculum (1:10 and 1:100 ratio of IsoF:test strain) the growth inhibitory effect was drastically reduced and in some cases even abolished (Fig 19). This tendency was also observed with other proteobacteria. Taken together, these results demonstrate that *P. putida* IsoF employs a dose- and contact-dependent inhibition system to impair growth of potential competitors.

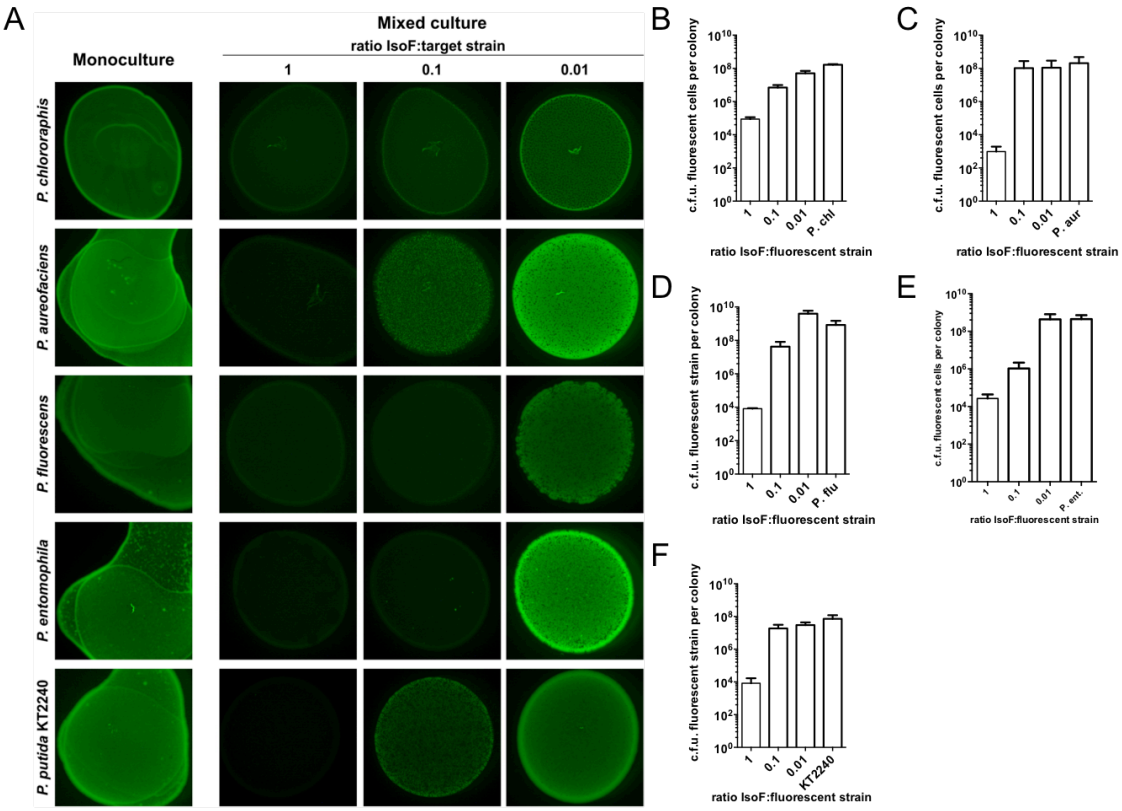

Figure 19. IsoF antagonism is doses dependent

IsoF and gfp-tagged pseudomonads were mixed at different ratios (IsoF:test-strain) on ABC medium (A) Fluorescence images of growth competition assays between the indicated GFP-labeled *Pseudomonas* and IsoF was monitored. Colony forming units (c.f.u.) of gfp-tagged (B) *P. chlororaphis,* (C) *P. aureofaciens,* (D) *P. fluorescens,* (E) *P. entomophila,* (F) *P. putida* KT2240, were monitored in competition with IsoF.

4.2.3 Quorum sensing, putisolvin and the large extracellular proteins LapA and LapF are not required for contact-dependent antagonism in *P. putida* IsoF.

Quorum sensing (QS) has been identified as an important regulator for contact-mediated antagonism in several bacteria, including *A. hydrophila* (Khajanchi et al., 2009), *P. aeruginosa* (Sana et al., 2012), *V. parahaemolyticus* (Wang et al., 2013) and *V. cholera* (Shao and Bassler, 2014). Since IsoF contains the *ppuI-rsaL-ppuR* QS system (Steidle et al., 2002) we tested the *ppuI* deficient mutant F117 (deficient in AHL production) for contact-dependent inhibition of *P. Putida* KT2240. IsoF produces the cyclic lipopeptide (CLPs) biosurfactants putisolvin I and II (Kuiper et al., 2004), which were shown to be associated with the bacterial cell envelope (Cárcamo-Oyarce et al., 2015). In addition to their biosurfactant properties, the CLPs often exhibit antibacterial activity (de Bruijn et al., 2007; Raaijmakers et al., 2006). We therefore included mutant PL11, which is mutated in the putisolvin biosynthetic gene *psoA* (Cárcamo-Oyarce et al., 2015), to test the involvement of putisolvin in the contact-dependent antagonism of IsoF. Moreover, IsoF possesses two large surface proteins, LapA and LapF, which have been reported to mediate cell-surface or cell-cell contacts (Martínez-Gil et al., 2010). We employed a mutant defective in the LapA transporter *lapB* and a *lapF* mutant to explore their possible roles in the antagonism diplayed by IsoF. Our results indicate that, under the conditions tested, neither QS, putisolvin, LapA nor LapF are required for the antagonistic activity of *P. putida IsoF* against *P. putida* KT2240 (Fig 20)

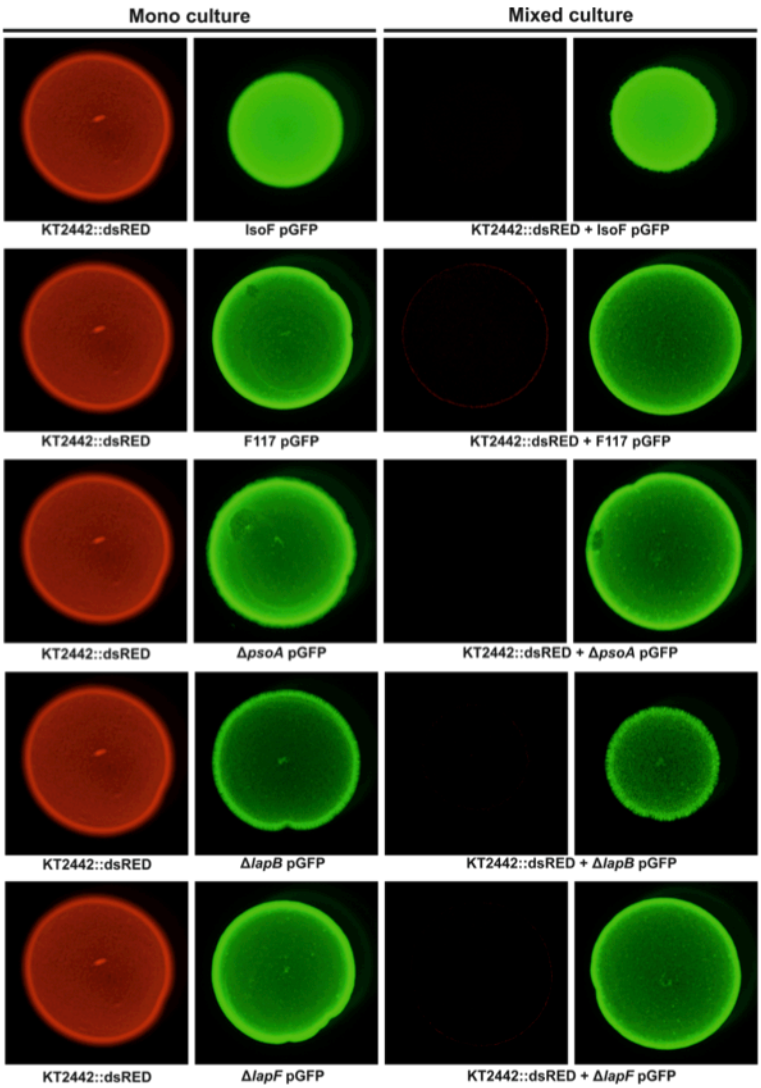

Figure 20. Quorum sensing, putisolvin and Lap proteins are not required for contact-dependent antagonism in IsoF

4.2.4 Contact-dependent antagonism is mediated by T6SS.

The presence of a T6SS has been reported in a number of Gram-negative bacteria (Murdoch et al., 2011; Russell et al., 2011; Russell et al., 2013). These systems utilize a bacteriophage-like apparatus to deliver protein effectors into diverse target cells (Silverman et al., 2012). Although *in silico* analyses have identified a T6SS in different *P. putida* strains (Barret et al., 2011; Boyer et al., 2009; Ye et al., 2014) their biological importance is unknown. We therefore engineered a mutant in the *vgrG* gene, the product of which is predicted to be part of the puncturing device of the T6SS, to evaluate the role of this T6SS system in the observed contact inhibition. Our results indicate that, in marked contrast to IsoF, a *vgrG* mutant does not display any measurable antagonism against the bacterial strains tested, thus strongly suggesting that this T6SS is necessary for the observed antagonism of IsoF (Fig 21).

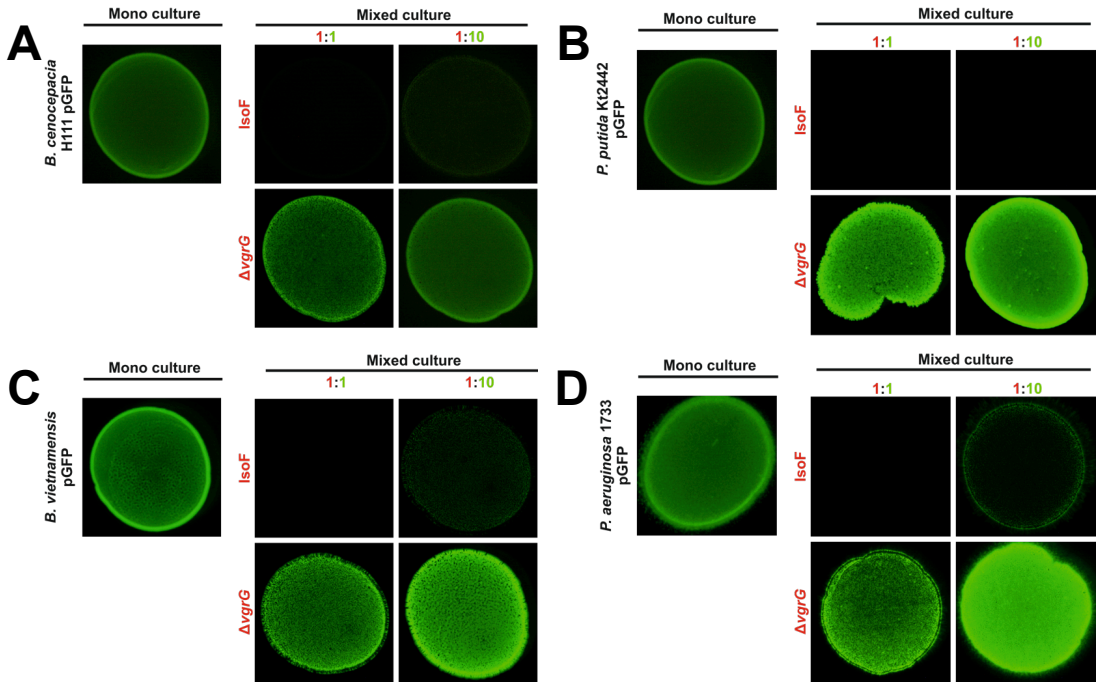

Figure 21. Contact-dependent antagonism is mediated by T6SS

When incubating (A) *B. cenocepacia* H111, (B) *P. putida* KT422, (C) *B. vietnamensis* and (D) *P. aeruginosa*; with the T6SS-mutant vgrG does not display antagonism against the bacterial strains tested as observed in co-incubation with IsoF

4.2.5 The IsoF T6SS system is required for biofilm defense and invasion

To investigate the role of the T6SS in isoF in mixed-species biofilms we used two different experimental settings. In the first setting, a two-species mixed biofilm was cultured by inoculating with equivalent numbers of cells (Fig 22). After 22h of cultivation, IsoF dominated the biofilms, as indicated by a 3.2-fold larger biofilm area and a 6.7-fold greater biofilm volume relative to KT2440 (Fig 22, compare C and D). To obtain visual evidence for the interactions between the two strains, we monitored the fate of a KT2240 cell cluster. In addition to the approximately 20% reduction in covered area and biovolume, we also observed dead cells at positions where the two strains were in direct contact, suggesting that upon surface contact IsoF had killed the KT2440 cells. In the second setting, the invasion of a preformed *P. putida* KT2240 monospecies biofilm was investigated (Fig 23). To this end flow-through cells with a 72 h old KT2240 biofilm were seeded with IsoF cells and the ability of IsoF to colonize the pre-existing biofilm was monitored (Fig 23). After 12h post-inoculation, microcolonies of IsoF were readily visible. We then quantified the areas and volumes of both strains within the biofilm. Our results demonstrate that IsoF has a T6SS-dependent negative impact on both the volume and the area occupied by KT2240, suggesting that IsoF uses the T6SS as an offensive weapon to invade existing biofilms.

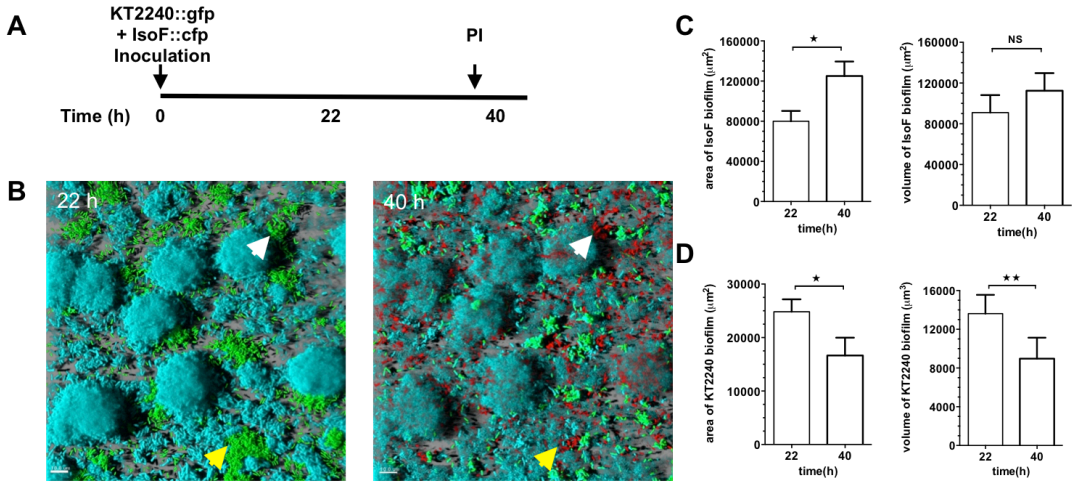

Figure 22. IsoF displaces KT2240 in mixed biofilms.

(*A*) Flow cells were inoculated with a 1:1 mixture of *P. putida* IsoF–cfp and *P. putida* KT2240–gfp. Each fluorescent signal was followed by CLSM. (B) Representative images of mixed biofilms. Biomass and Covered area of (C) *P. putida* KT2240 and (D) *P*. *putida* IsoF biofilms were assessed during the experiment.

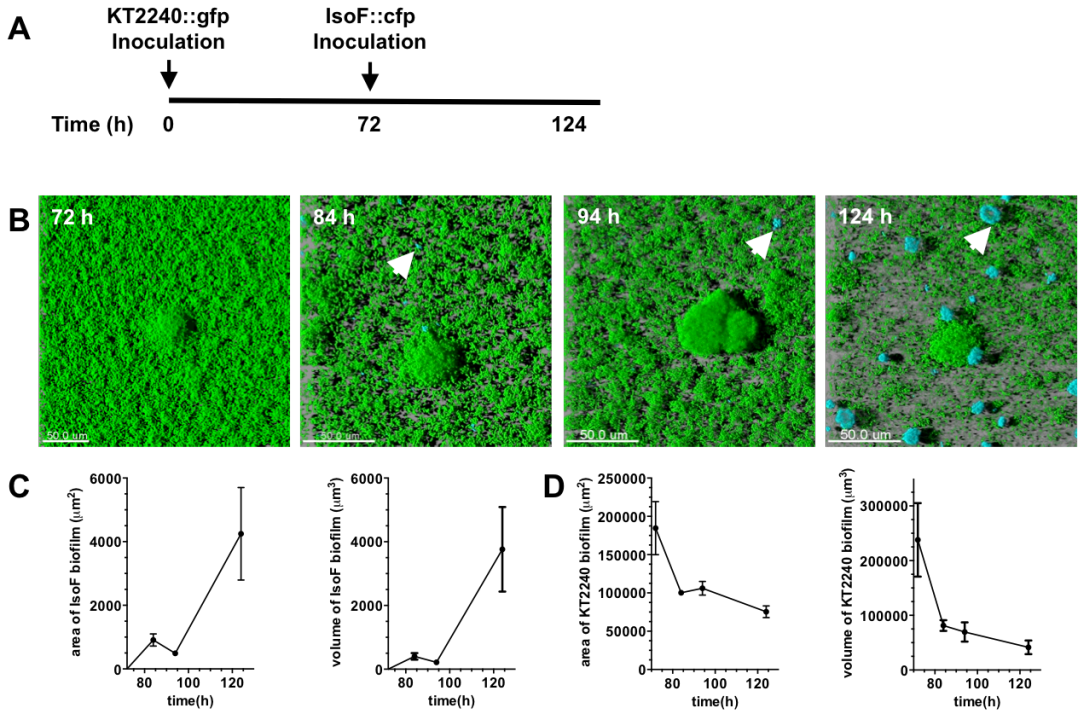

Figure 23. IsoF invades preexisting KT2240 biofilm

(*A*) Flow cells were inoculated with *P. putida* KT2240–gfp, after 72h *P. putida* IsoF–cfp was inoculated to the system. Each fluorescent signal was followed by CLSM. (B) Representative images of mixed biofilms. Biomass and Covered area of (C) *P. putida* KT2240 and (D) *P. putida* IsoF biofilms were assessed during the experiment.

4.3 Discussion

In natural environments bacteria compete for limited resources and space (Hibbing et al., 2010). Various competition strategies exist that aim at decreasing either the survival or reproduction of competitors, often by depriving competitors for nutrients (Wandersman and Delepelaire, 2004) or by producing antimicrobials (Raaijmakers et al., 2002). In a heterogeneous environment as represented by a natural biofilm (Stewart and Franklin, 2008), the ability to colonize and persist in the most favorable locations represents another competition strategy (Nadell and Bassler, 2011; Schluter et al., 2015). Due to the physical confinement in biofilms, contact-dependent inhibition systems are likely to be important for the interaction of bacteria within the biofilm matrix.

In this work, we established that *P. putida* IsoF uses a T6SS to antagonize a wide range of proteobacterial species. The broad range of bacterial targets is consistent with previous reports on the T6SSs of *P. aeruginosa* (Hood et al., 2010), *Burkholderia thailandensis* (Schwarz et al., 2010), *Vibrio cholerae* (MacIntyre et al., 2010) and *Serratia marcescens* (Murdoch et al., 2011). Interestingly, the IsoF T6SS was found to be particularly effective against soil- and rhizosphere-associated pseudomonads, including *P. putida* KT2240, *P. chlororaphis, P. aureofaciens*, *P. fluorescens* and *P. entomophila*, suggesting that the T6SS plays an important role for the ecology of the rhizosphere microbial communities, where the bacteria were shown to form dense aggregates (Philippot et al., 2013; Steidle et al., 2002)..

Contact-dependent growth inhibition appears to be a fundamental mechanism for persistence of bacteria in mixed biofilms. Previous work has shown that T6SSs are involved in bacterial attachment and biofilm maturation (Khajanchi et al., 2009; Tian et al., 2015). In this study we show that the T6SS of IsoF was capable of effectively displacing competitors that were in direct contact in mixed biofilms. Similar results were previously described for *B. thailandensis* when grown in mixed biofilms with *P. putida* (Schwarz et al., 2010).

Bacteria living in biofilms are much more resistant to various stresses, including predation, the immune system, antimicrobials and biocides, than planktonic cells (Draper et al., 2015; Flemming and Wingender, 2010; Hall-Stoodley et al., 2004; Stewart and Costerton, 2001).Biofilms have also been shown to be resistant to invasion by non-resident bacterial cells (Nadell et al., 2015; Rao et al., 2005). Nadell

et al. (2015) demonstrated that although planktonic *V*. *cholerae* cells can temporarily colonized the outer layers of a pre-established biofilm, the extracellular matrix, particularly the secreted protein RbmA, protects the biofilm from invasion. Here we show that *P. putida* IsoF is capable to invade and displace a major P. putida KT2440 biofilm. To our knowledge this is the first report that a T6SS is used as an offensive weapon to invade existing biofilms and is not only required for persistence within a biofilm. . Additional work will be required to unravel the molecular mechanism of T6SS-mediated killing of bacteria in *P. putida* IsoF and to investigate whether T6SSs are generally used by bacteria for biofilm invasion.

Our results extend the understanding of the complexity of bacterial interactions and dynamics in multispecies biofilms.

4.4 Materials and Methods

4.4.1 Strains, Media and Culture Conditions

Bacterial strains and plasmids used in this study are listed in Table S1**.** All strains were grown aerobically in Luria–Bertani (LB) at 30°C (*Pseudomonas putida*) or 37°C (*Escherichia coli*). *P. putida* transconjugants were isolated on Pseudomonas Isolation Agar (PIA) at 30°C. In most experiments *P. putida* strains were grown in modified AB medium supplemented with 10 mM sodium citrate (Heydorn et al., 2000) (referred henceforward as ABC medium). Antibiotics were added as required at final concentrations of: for *E. coli*: 100 µg ml^{-1} ampicilin, 50 µg ml^{-1} kanamycin, 10 µg ml^{-1} gentamycin; for *P. putida*: 100 µg ml^{-1} kanamycin and 20 or 50 µg ml^{-1} gentamycin

4.4.2 Construction of *P. putida* IsoF mutants

The T6SS mutant vgrG was generated as follows: an internal *psoR* fragment was PCR amplified using the primers vgrGF (5'-AAGGCGACACAGGTTTTTGC-3') and vgrGR (5'-TGATGTTTCAGGTCACTGGCT-3') and cloning the PCR product into pCR2.1 TOPO. The resulting plasmid, pCR2.1 vgrG*,* was mobilized into *P. putida* IsoF by standard electroporation (Choi and Schweizer, 2006) and gene replacement mutants were selected on PIA medium containing 50 µg ml^{-1} kanamycin. The genetic structures of all mutants constructed were confirmed by PCR and sequence analysis using the primer vgrG_out (5'-GCACATCATCGCCTTCCACT-3').

4.4.3 Construction of fluorescently labeled strains

The mini-Tn7 system was utilized to integrate green fluorescent protein (GFP), cyan fluorescent protein (CFP) and red fluorescent protein (dsRED) expression cassettes into the chromosome of *P. putida* IsoF, *P. chlororaphis, P. aureofaciens, P. fluorescens, P. entomophila, P. putida* KT2240 (Lambertsen et al., 2004). The conjugative donor strain *E. coli* S17-1 was transformed with the mini-Tn7 and the helper plasmid pUX-BF13. Mini-Tn7-labeled strains were obtained after strain triparental mating (Choi and Schweizer, 2006).

4.4.4 Competition assays

Overnight cultures of were adjusted to an OD600nm of 0.1 and mixed at different ratios (IsoF:test-strain, 1:1, 1:10, 1:100) on ABC minimal medium or LB depending the strains growth requirements. The fluorescence of tagged-strains was then monitored by means of fluorescence microscopy. The bacterial growth was assessed by c.f.c. counts.

4.4.5 Cultivation of biofilms, microscopy, and image analysis

Biofilms were grown in flow cells supplied with ABC medium. The flow system was assembled and prepared as described previously (Christensen et al., 1999). Briefly, the flow channels were inoculated with *P. putida* cultures in minimal medium supplemented with citrate as carbon source. The medium flow was kept at a constant rate of 0.2 mm s–1 by a Watson-Marlow 205S peristaltic pump. The incubation temperature was 30°C. Microscopic inspection and image acquisition were performed using a confocal laser scanning microscope (CLSM) (DM5500Q; Leica) equipped with a x40/1.3 or a x63/1.4 oil objective. Captured images were analyzed with the Leica Application Suite (Mannheim, Germany) and the Imaris software package (Bitplane, Switzerland). Images were prepared for publication using CorelDraw (Corel Corporation) and PowerPoint (Microsoft) software.

4.5 References Results III

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5 General discussion

The results presented in this thesis address some hot topics in the field. In addition to the discussions in each of the chapters of this thesis I would like to highlight some more general aspects in the following:

5.1 Heterogeneity in QS induction

The QS paradigm established that once a bacterial population has reaches a certain density, all members of the population switch to a “quorum mode” (Bassler and Losick, 2006; Fuqua et al., 1994; Waters and Bassler, 2005; Winans and Bassler, 2008). This implies that all members of the community should have identical levels of AHLs and regulator molecules. Just in that situation all cells can synchronize their activities, giving rise to coordinated behaviours. However, It is well establish that under normal circumstances bacterial populations are heterogeneous with respect to gene expression even among cells growing under controlled and stable environmental conditions (Avery, 2006; Elowitz et al., 2002).

Previous work showed heterogeneiety in the induction of the QS response, both at low (Boedicker et al., 2009), and at high cell density (Anetzberger et al., 2009; Anetzberger et al., 2012; Perez and Hagen, 2010; Perez et al., 2011; Pradhan and Chatterjee, 2014). Importantly, in the framework of this thesis I could provide the first example that QS heterogeneity serves as a mechanism to trigger a self-directed behavior of individual cells. A recent study investigated the underlying mechanisms of small molecule-driven cell-to-cell communication systems to serve for autocrine or paracrine signaling (Youk and Lim, 2014). By manipulating relevant circuit elements (i.e. receptor levels, signal secretion rate, positive feedback and signal degradation), the authors examined whether the cells responded to their own secreted signal (self-communication or autocrine signaling) or to the signal secreted by neighbors (neighbor communication or paracrine signaling). The authors show that at low cell density, high intercellular receptor levels and a strong positive feedback in the sensing circuitry self-communication is favoured over neighbor-communication in the population. These results may mimic the situation found in young IsoF biofilms, where AHL production appears to be mainly used for self-induction. Further studies

will be required to explore the role of self-induction versus neighbor communication in biofilm development of *P. putida* IsoF.

5.2 Extending the QS paradigm

By studying QS induction at the single cell level, it showed that QS signals were stochastically produced in early stage biofilms of *P. putida* and they acted mainly as self-regulatory signals triggering asocial motility of induced cells out of microcolonies. These results added a new facette to the classical view of QS. While the QS paradigm is thought to provide a mechanism that enables cells to coordinate theirs behaviors at high population densities, it showed that QS can also do the opposite, namely to trigger uncoordinated self-directed behavior at low cell density. The reasons for stochastic induction of QS in IsoF were discussed in detail in chapter II and may result from high sensitivity to AHLs (Kaplan and Greenberg, 1985), intracellular AHLs self-activation (Buroni et al., 2009; Chan et al., 2007; Pearson et al., 1999) and/or physiological differences between biofilm cells (Folsom et al., 2010; Stewart and Franklin, 2008). I proposed that QS is more complex than previously thought, as the traits being induced by QS can cover the entire continuum from a cooperative public good trait that generates benefits to others (e.g. elastase production (Diggle et al., 2007)), to extra-cellular traits that mostly generate self-directed benefits (e.g. putisolvin production), to entirely intra-cellular traits that solely provide benefits to the producer.

5.3 Ecological interpretations of QS-regulated traits

In agreement with the QS paradigm, it is assumed that in general QS regulates the production of public goods that benefit the entire population (Darch et al., 2012; Schuster et al., 2013). Even when QS regulates the production of private goods (i.e. intracellular enzymes), it has been interpreted to be beneficial for the community as a method to stabilize cooperative behaviors by avoiding the emergence of cheaters in the population (Dandekar et al., 2012).

A cooperative behavior is considered to provide a benefit to another selected individual (recipient), whereas a non-cooperative behavior provides a benefit exclusively for the same individual (West et al., 2007). The definition of benefit (i.e. increase in fitness) is commonly done in light of evolution biology theories.

Parameters related to bacterial growth (i.e. optical density, c.f.u. counts, growth rate) are often compared among different populations (wild type vs a mutant, producer vs non-producer, etc) to who profits most from public goods (Brown and Johnstone, 2001; Diggle et al., 2007; Matz, 2011; Oliveira et al., 2014; West et al., 2006). While this is a valid approach it usually does not consider the importance of phenotypical heterogeneity within bacterial populations (Avery, 2006; Dhar and McKinney, 2007; Lidstrom and Konopka, 2010). Future studies should therefore also consider the heterogeneity in AHL production and receptor availability for a proper interpretation of who is in fact benefiting from the production of QS-controlled goods or by cheating.

In a recent comprehensive review the general principles governing auto-induction in bacteria were analyzed (Hense and Schuster, 2015). The authors suggest that the escape of individual cells from biofilms triggered by QS could also be interpreted as an altruistic behavior (which is costly to the actor and beneficial to the recipient) that eventually would increases the fitness of the resident population by reducing starvation stress. On the contrary, I proposed that in IsoF young biofilms, QS triggers a non-cooperative behavior, allowing the induced cells to reach new resources faster than the competitors. This notion is in also in agreement with the fact that bacterial dispersal is induced as consequence of nutrient limitation within the biofilm community (Gjermansen et al., 2010; Gjermansen et al., 2005; Gjermansen et al., 2006; Newell et al., 2011; Newell et al., 2009).

5.4 Evolution of QS systems in bacteria

The evolution and acquisition of QS cluster genes by different bacterial linages has been subject of several reports (Kimura, 2014; Lerat and Moran, 2004; Nasuno et al., 2012). Horizontal gene transfer has been suggested as a main source of *luxIR* systems some Gram-negative bacteria (Lerat and Moran, 2004). Likely, this is also the case for those *P. putida* strains that harbor the *ppuIR* gene cluster. Knowledge on how this cluster was acquired and from where it originated is has not been elucidated. *P. putida* strains, particularly IsoF, may therefore be a valuable case of study for a better understanding of their evolution of QS systems.

5.5 Multispecies biofilm dynamics

The vast majority of biofilms research is focused on monospecies biofilms. However, it is well accepted that in most natural settings bacteria exist in multispecies biofilms (Costerton et al., 1999; Danhorn and Fuqua, 2007; Hall-Stoodley et al., 2004). Bacteria face a constant battle for space and resources in such communities (Hibbing et al., 2010). Because of the close association of bacteria within biofilms they have developed strategies to promote beneficial interactions (Elias and Banin, 2012) and to prevent detrimental associations (Rendueles and Ghigo, 2012). The role of contact-dependent interactions has only recently emerged as an important mechanism in the development of mixed biofilms (Hayes et al., 2010; Russell et al., 2014). Within this thesis I showed that *P. putida* IsoF used a T6SS-to as an offensive weapon to invade preexisting biofilms of related *P. putida* strains. This may resemble the situation found in nature where surfaces are normally already colonized by biofilms, which prevents settlement of invaders. Further investigation about the environmental factors that trigger T6SS activity, the range of antagonist activity as well the ecological implications of this mechanism of competition are essential for a better understanding of the complexity of bacterial interactions and dynamics in multispecies biofilms.

5.6 References General Discussion

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